PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 59131-A-PCT	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/15621	International filing date *(day/month/year)* 07 JUNE 2000	(Earliest) Priority Date *(day/month/year)* 07 JUNE 1999
Applicant THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 7 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [ ] furnished subsequently to this Authority in written form.
   [ ] furnished subsequently to this Authority in computer readable form.
   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has b een furnished.
2. [ ] **Certain claims were found unsearchable** (See Box I).
3. [X] **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. _____
   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/15621

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please see extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-25, 29-38, 49, 53 and 54

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

A new primer, 5′-ACACGACGAACCGGCAGGTG-3′, was derived from this sequence and used in combination with a λ ZAP–specific primer (Stratagene) to amplify a portion of the *ZMM2* (*ucsd78b*) transcript from our ear and tassel cDNA libraries (*19*). A full-length cDNA was subsequently isolated from the tassel library by screening at high stringency with a probe from the 3′ end of the partial clone. RFLP analysis on recombinant inbreds confirmed that the correct locus had been obtained.

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24. J. L. Riechmann, B. A. Krizek, E. M. Meyerowitz, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4793 (1996).
25. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. T. A. Kiesselbach, *The Structure and Reproduction of Corn* [*Univ. Nebr. Agric. Exp. Stn. Res. Bull. 161* (1949)].
27. Analysis of RNA expression was performed as described in Fig. 1. A 500–base pair (bp) Hpa I–Nde I fragment was used as the *ZAG1* probe in Fig. 4B. The *ZMM2*-specific probe (see below) was a 400-bp Xho I fragment isolated from the 3′ end of the cDNA. Both probes were labeled to comparable specific activities. The faint *ZMM2* hybridization detected in endosperm is likely a result of nucellar tissue, which often contaminates endosperm tissue obtained from early developmental stages. RNA from immature ears and tassels was isolated from developing inflorescences that were <3 cm in length. Hybridization with a tubulin probe produced signal in every lane.

28. We thank T. Fox and M. Albertson for use of their tassel cDNA library. Supported in part by postdoctoral fellowships from the North Atlantic Treaty Organization and Ministerio de Educación y Ciencia (to M.M.) and from the Pioneer Discovery Research Program (to R.B.M.) and by grants from the U.S. Department of Agriculture and the University of California Biotechnology and Research Foundation (to R.J.S. and M.F.Y.).

5 July 1996; accepted 13 September 1996

CRNF, a Molluscan Neurotrophic Factor That Interacts with the p75 Neurotrophin Receptor

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A 13.1-kilodalton protein, cysteine-rich neurotrophic factor (CRNF), was purified from the mollusk *Lymnaea stagnalis* by use of a binding assay on the p75 neurotrophin receptor. CRNF bound to p75 with nanomolar affinity but was not similar in sequence to neurotrophins or any other known gene product. CRNF messenger RNA expression was highest in adult foot subepithelial cells; in the central nervous system, expression was regulated by lesion. The factor evoked neurite outgrowth and modulated calcium currents in pedal motor neurons. Thus, CRNF may be involved in target-derived trophic support for motor neurons and could represent the prototype of another family of p75 ligands.

The survival, differentiation, and plasticity of vertebrate neurons are influenced by neurotrophic factors, the best characterized of which are the neurotrophins (*1*). Invertebrate neuronal systems have provided useful models for development and regeneration of the nervous system; however, efforts to clone neurotrophin or neurotrophin receptor–like sequences in invertebrates have so far been unsuccessful (*2*). Indeed, it has been argued that neurotrophic factors may be a comparatively late addition to the arsenal of mechanisms controlling nervous system development and regeneration, and that such factors may be required only in long-lived organisms with complex nervous systems (*2*). To examine the existence of neurotrophin-related molecules in an invertebrate, we focused on the snail *Lymnaea stagnalis*, which is used as a model in cellular and molecular neuroscience (*3*, *4*). *Lymnaea* hemolymph or central nervous system (CNS)–conditioned medium (CM) evoke neurite outgrowth from snail motor neurons. This activity can be mimicked by murine nerve growth factor (NGF), which furthermore has acute effects on calcium currents in *Lymnaea* neurons (*4*).

Initial attempts to clone a *Lymnaea* neurotrophin homolog by polymerase chain reaction (PCR) on the basis of conserved regions of vertebrate neurotrophins (*5*) were not successful. Therefore, we adopted a functional approach to target neurotrophin family members. All neurotrophins interact with two receptor types—ligand-specific receptor tyrosine kinases of the Trk family (*6*) and a shared receptor termed p75 (*7*). Starting from the premise that a putative molluscan neurotrophin homolog might also bind to the p75 receptor, we assayed *Lymnaea* CM and hemolymph for inhibition of binding of ^{125}I-labeled NGF to A875 human melanoma cells, which express high levels of p75 but no Trk receptors. Hemolymph and CM-derived fractions inhibited NGF binding to p75 in a dose-dependent manner (Fig. 1A). Further fractionation revealed that NGF-displacing fractions from both CM and hemolymph had identical chromatographic elution properties and contained an almost identical protein mass of 13.1 kD.

The higher amounts of the 13.1-kD protein in hemolymph enabled its purification to homogeneity from a pool of 7 liters of snail hemolymph, using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) to monitor the purification (*8*). The final purified fraction (Fig. 1B) contained a major component of 13.1 kD and a minor component of 13.97 kD, as analyzed by MALDI-MS (Fig. 1C). Internal and NH_2-terminal peptide sequences of both proteins were identical and novel. These sequences served to design primers for PCR and eventual isolation of a cDNA clone (*9*), which encoded an open reading frame of 121 amino acids, including an 18-residue putative signal sequence followed by a mature product of 103 amino acids containing all the peptide sequences previously obtained (Fig. 1E). The sequence was not significantly similar to any known protein or DNA sequence in the public databases. The protein molecular mass predicted from the cDNA sequence is 12.5 kD, which is close to the measured masses of the purified CRNF isoforms and suggests that the latter may arise from different extents of glycosylation on the single consensus *N*-glycosylation site (Fig. 1E). The sequence contained a high number of cysteine residues, comprising over 10% of the protein, hence the name CRNF (cysteine-rich neurotrophic factor).

An anti-peptide antiserum was raised (*9*) against a synthetic peptide based on the COOH-terminal region of the CRNF se-

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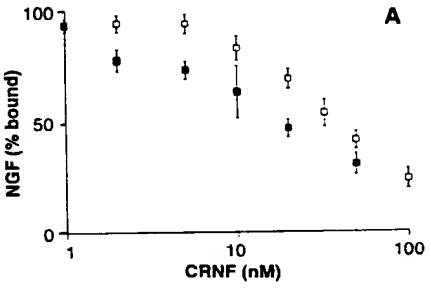
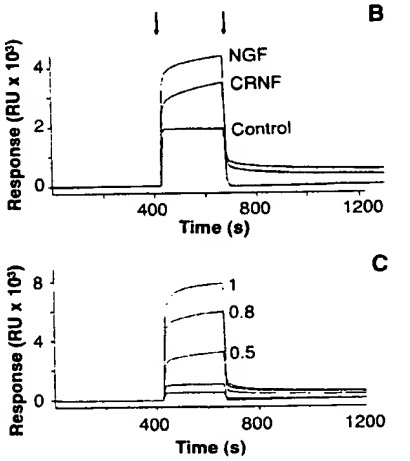
quence. Immunoreactive bands with apparent electrophoretic mobilities of 14 to 16 kD were observed upon protein immunoblotting of *Lymnaea* foot tissue homogenates (Fig. 1D). This antiserum was subsequently used to monitor production of recombinant CRNF in baculovirus-infected insect cells (9). Recombinant CRNF was readily produced in baculovirus-infected insect cells, but was not secreted, and could be identified in cell lysates as two major immunoreactive bands with apparent molecular masses of 18 and 24 kD (Fig. 1D). No CRNF immunoreactivity could be found in mock-infected cells. The larger apparent mass of the protein produced in baculovirus could be due to incomplete processing of the signal peptide in the insect cells or to a higher extent of glycosylation.

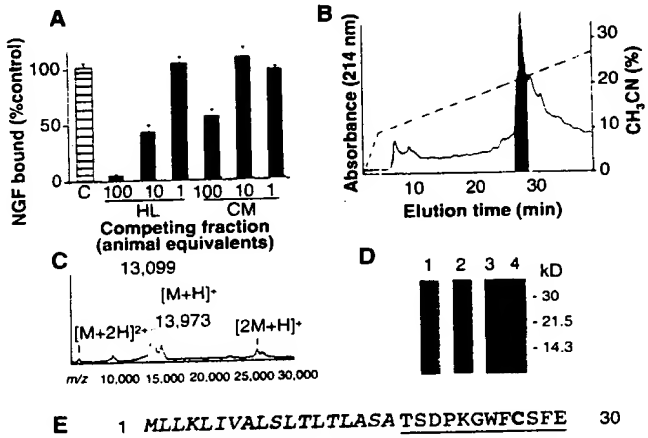
Both native and recombinant CRNF inhibited binding of ^{125}I-NGF to p75 in a dose-dependent manner with measured median inhibitory concentrations (IC_{50}'s) of approximately 15 nM for the native protein and 45 nM for recombinant CRNF (Fig. 2A). CRNF displacement of NGF from p75 could be mediated by a number of different mechanisms, including competitive binding of CRNF to p75 or a direct interaction between CRNF and NGF. We used surface plasmon resonance (*10*) to determine directly whether CRNF can interact with NGF or with a soluble p75 extracellular domain (Sp75) (*11*). NGF or CRNF were immobilized separately on BIAcore sensor chips, and these chips were subsequently used to monitor binding of the other ligand or of Sp75. No interaction between NGF and CRNF was observed. In contrast, Sp75 bound comparably to both immobilized NGF or CRNF, whereas no binding was observed on control chips (Fig. 2B). Titration of Sp75 binding to CRNF (Fig. 2C) enabled calculation of k_{off} and k_{on} from the dissociation and association phases of the curves, respectively. The apparent equilibrium dissociation constant (K_d) subsequently calculated from these kinetic measurements was 50.9 ± 9.7 nM, which is in the same range as the IC_{50} for competitive displacement of NGF from A875 cells.

In order to gain insight into possible physiological roles of CRNF, we examined its mRNA expression in various tissues of the adult snail by ribonuclease protection assays (RPAs). High CRNF mRNA expression was almost exclusively restricted to the foot, with low levels in mantle tissue (Fig. 3A). In addition, CRNF expression could be induced by mechanical lesion of CNS explants in vitro (Fig. 3B), suggesting a role for CRNF in injury repair. In situ hybridization was performed to localize CRNF expression sites in the peripheral tissues comprising the foot. Intense labeling was observed in a distinct layer of large globular subepithelial cells (Fig. 3C). This region of the foot has extensive arborization from the pedal nerves on the way to their target cells (*12*).

The highly restricted pattern of expression of CRNF mRNA in adult foot might indicate trophic roles for this molecule on peripheral and pedal neurons innervating the foot. Adult *Lymnaea* neurons are not dependent on exogenous trophic factors for survival in vitro, and furthermore, developmental programmed cell death has not been observed in molluscan CNS (*13*). However, trophic activity in molluscan neurons can readily be monitored as neurite outgrowth in isolated neuronal cultures (*3*, *4*). CRNF activities were examined, using *Lymnaea*







E

1 *MLLKLIVALSLTLTLASA* TSDPKGWFCSFE 30
31 CNDWNQLEMACQKEKDCFEKTNNQFTNETV 60
61 ACMQPCWDLGIPCRTQCYKVYDQCTSDCPY 90
91 TVPNPYECFTTCFKERSPRSNLKYPKQILM 120
121 Q•

Fig. 1. Identification and purification of CRNF. (**A**) *Lymnaea* hemolymph (HL, gray bars) and CM-derived fractions (black bars) inhibit binding of ^{125}I-NGF to p75 receptors on A875 melanoma cells in a dose-dependent manner. Striped bar indicates control. (**B**) Final reverse-phase high-performance liquid chromatography step in the purification of CRNF, Vydac C4, with a flow of 1 ml/min. Gradient of acetonitrile in aqueous 0.1% TFA is shown by dashed line; CRNF fraction is indicated by shading under the peak. (**C**) MALDI-MS on the purified peak reveals two protein masses of 13.10 and 13.97 kD. (**D**) SDS–polyacrylamide gel electrophoresis of purified CRNF under reducing conditions reveals two bands upon Coomassie staining after blotting (lane 1). An antiserum to CRNF peptide identified a 14- to 16-kD band on protein immunoblots of extracts of *Lymnaea* foot tissues (lane 2). Insect cells infected with a CRNF-recombinant baculovirus revealed immunoreactive bands of higher apparent mass in insect cell lysates (lane 4), but not in conditioned medium (lane 3). Molecular size markers are indicated. (**E**) Amino acid sequence of CRNF, as predicted from the cDNA clone. The putative signal sequence is in italics, cysteine residues are in bold, one consensus site for N-linked glycosylation is indicated by an asterisk, and the stop codon is indicated by a dot. Sequences corresponding to the peptides previously obtained by Edman degradation are underlined. Single-letter amino acid abbreviations are as in (*18*).

Fig. 2. Binding of CRNF to the p75 neurotrophin receptor. (**A**) Inhibition of ^{125}I-NGF binding to A875 cells (*19*) by native (solid squares) and recombinant CRNF (empty squares). (**B**) Surface plasmon resonance measurement of the interaction of 0.5 μM soluble p75 extracellular domain (Sp75) with control chips, and chips on which NGF or CRNF was immobilized (*20*). Arrows indicate start and ending of Sp75 application. Note the association and dissociation curves observed on NGF or CRNF chips, in contrast to the unspecific mass effect seen on the control chip. RU, resonance units. (**C**) Titration of Sp75 interaction with immobilized CRNF. Concentrations of Sp75 in μM are indicated for each trace.

pedal A motor neurons (PedA), which are a potential target population for this factor. Purified CRNF evoked neurite outgrowth from PedA neurons in vitro (Fig. 4, A through C), with responses including neurons that displayed multiple short neurites tipped with small growth cones (Fig. 4B), whereas other neurons formed large growth cones that approached the cell body in diameter (Fig. 4C). This response peaked within 24 hours and was dose dependent, with a maximal effective concentration of 25 pM (Fig. 4D). The lower response seen at higher doses of CRNF is reminiscent of the bell-shaped dose-response relationships previously reported for several vertebrate trophic factors in outgrowth assays [for example, (*14*)].

Murine NGF enhances high-voltage activated (HVA) calcium currents in *Lymnaea* Parietal A motor neurons (ParA), within seconds of application (*4*). Therefore, we examined the effects of CRNF on HVA calcium currents in ParA neurons. Superfusion of ParA neurons in vitro with 1 nM CRNF modulated the HVA calcium currents in 50% of the cells tested (n = 8). In ParA cells expressing both fast- and slow-inactivating calcium currents, CRNF enhanced the peak calcium current (Fig. 4E). The current-to-voltage relation (Fig. 4F) illustrates that the effect of CRNF was primarily on the fast-inactivating current, whereas the slow-inactivating current remained largely unaffected. In contrast to previous observations with murine NGF, the effects of CRNF on the HVA calcium currents did not reverse within a few minutes of washing. Thus, CRNF differentially modulated the various components of HVA calcium currents expressed in *Lymnaea* motor neurons, and may play a role in plasticity-related processes in *Lymnaea* CNS.

We propose that CRNF is an invertebrate neurotrophic factor (*15*) because it is expressed in a restricted pattern and, at low levels, it has trophic and plasticity-related activities at low concentrations on potential target neurons and was identified on the basis of its interaction with the p75 neurotrophin receptor. CRNF shares no obvious sequence similarity with mammalian neurotrophins, suggesting that properties in common between these two families may have arisen from convergent evolution. Alternatively, as our data do not rule out the possibility that neurotrophins exist in *Lymnaea*, CRNF may represent the prototype of a novel family of p75 ligands. A number of gene families important in axon guidance and wiring of the nervous system were first identified in invertebrates (*16*); therefore, the phyletic distribution of CRNF is of primary interest.

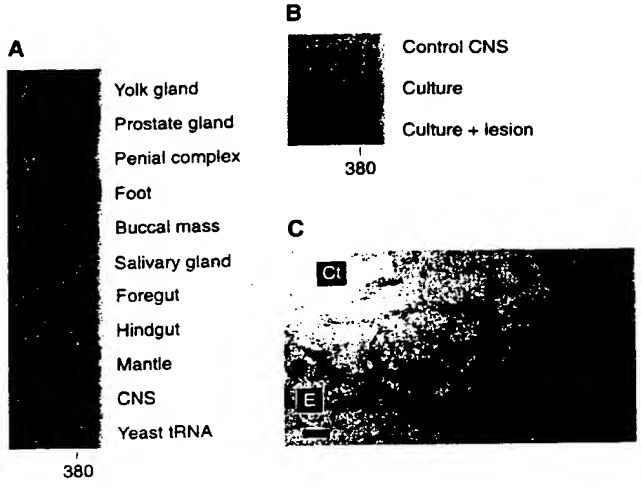


Fig. 3. Expression of CRNF mRNA in adult *Lymnaea* tissues. (**A**) RPA on 5 μg total RNA from different adult tissues revealed high expression of CRNF mRNA in the foot and lower levels in mantle tissue (overnight exposure) (*21*). Position of a 380-nucleotide band is indicated. (**B**) RPA analysis of CRNF mRNA in control CNS compared to CNS after 36 hours in culture and cultured CNS after crush lesion to the interganglionic connectives and commisures. We used 20 μg total RNA from each sample, and exposure time was 7 days. (**C**) Localization of CRNF mRNA expression in adult foot by in situ hybridization. Intense signal was observed in a layer of large subepithelial cells. No signal was detected using a control probe. (E, epithelial layer; Ct, connective tissue layer; scale bar, 50 μm.)

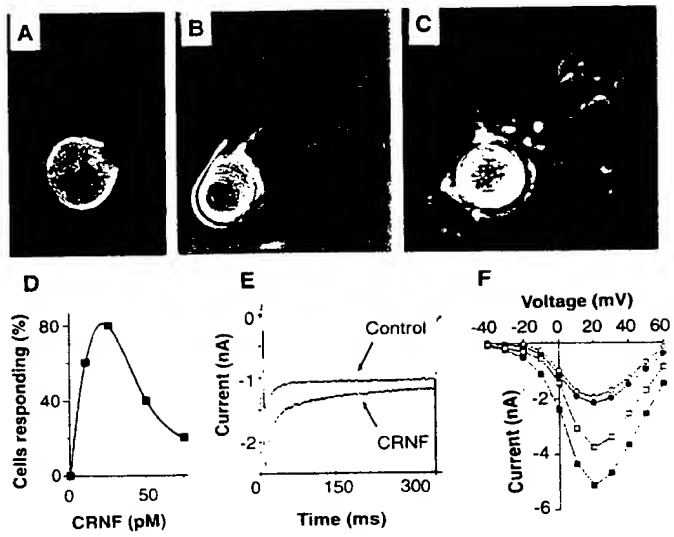


Fig. 4. Effects of CRNF on *Lymnaea* motor neurons. (**A** through **D**) CRNF evokes neurite outgrowth from cultured PedA motor neurons. (A) Control cultures showed no neurite extension. (B and C) Neurons developed processes and growth cones 24 hours after treatment with CRNF. (D) Dose-response relation of the number of cells exhibiting neurite outgrowth in response to CRNF (n = 6 for each data point). (**E** and **F**) Modulation of HVA calcium currents in Right Parietal A motor neurons by CRNF. (E) Calcium current recorded under whole-cell clamp techniques at a test potential of 0 mV (holding potential of −80 mV) before application of CRNF (control) and 10 min after the start of an application of 1 nM CRNF. The early peak current was markedly increased in the presence of CRNF. (F) Current-to-voltage relations of peak (squares) and late (circles) currents show that the effect of CRNF appears restricted to the peak current. Open symbols are peak and late current values prior to CRNF application and filled symbols are values 10 min after the start of CRNF application. Methods were as described (*4*).

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8. Hemolymph was milked from adult *Lymnaea* by collecting fluid ejected after tapping the foot. The final 7-liter pool of hemolymph represented approximately 16,000 animal milkings. Aliquots of 1 liter were processed as follows: (i) Ultracentrifugation for 2 hours at 120,000*g* to precipitate hemocyanin. (ii) Supernatant was applied to Polybuffer 94 (Pharmacia) equilibrated and washed with 20 mM tris (pH 7.9). Bound proteins were eluted by 1 M NaCl and desalted and concentrated with Poros 20 R1. [illegible] eluted

fractions were applied to linked Beckman SW2000-SW3000 molecular exclusion columns, equilibrated, and eluted with 0.5× phosphate-buffered saline/15% acetonitrile. (iv) Fractions eluting at apparent masses of 20 to 30 kD were loaded onto fast-flow reverse-phase Poros 20 R1 equilibrated at 4 ml/min in 15% acetonitrile/0.1% trifluoroacetic acid (TFA). After a 2-min wash, the bound proteins were eluted with a linear gradient from 15 to 60% acetonitrile in 2 to 24 min. (v) Fractions eluting from Poros R1 at 32 to 36% acetonitrile were refractionated on wide-pore Vydac C4 (250 mm by 4.6 mm, 5 μm particle size) at 1 ml/min, in a gradient of acetonitrile in 0.1% TFA (Fig. 1B). Amino acid sequence analysis was performed on blotted Coomassie stained bands, or reverse phase purified peptides from Endo-LysC digest, by automated Edman degradation on an Applied Biosystems 473A system. Methods for MALDI-MS were as described (*17*).

9. Degenerate oligonucleotide primers encoding all possible codons for TSDPKGWF (*18*) (sense, NH_2-terminal) and PYTVPNPY (*18*) (antisense, internal peptide sequence) were used for PCR on *Lymnaea* CNS cDNA. A 230–base pair (bp) product was cloned, sequenced, and used to screen a CNS cDNA library, resulting in isolation of a 472-bp cDNA clone. The nucleotide sequence of CRNF has been deposited in Genbank (accession number U72990). Mouse polyclonal antisera were raised against RSNLKYPKQILM (*18*) (residues 109 through 120 of the amino acid sequence). An expression construct was made by PCR amplification of the CRNF coding region, which was subcloned into pBAC (Clontech). Recombinant baculovirus were generated from the pBAC construct, using Clontech reagents according to manufacturer's instructions. Recombinant protein was produced in baculovirus-infected insect cells (*19*) and purified according to (*8*).
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17. C. R. Jiménez *et al.*, *J. Neurochem.* **62**, 404 (1994).
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. M. Rydén *et al.*, *EMBO J.* **14**, 1979 (1995).
20. Immobilization of NGF or CRNF to BIAcore CM5 sensor chips was done by amine coupling in 20 mM acetate buffer (pH 5.6 or 3.6). Binding of Sp75 to immobilized ligand was monitored in a BIAcore 2000 Biosensor (Pharmacia) at 20°C, with a flow of 5 ml/min, in Hepes-buffered saline. Kinetic analyses were done with BIAevaluation software, version 2.0 (Pharmacia).
21. Riboprobes for RPA and in situ hybridization were generated from linearized 380-bp subclones of CRNF cDNA in pCDNA3 (Stratagene). RPA was performed with equal amounts of total RNA, using RPAII reagents (Ambion). Equal loading was verified on ethidium bromide–stained gels and by parallel RPA with riboprobes for ubiquitously expressed *Lymnaea* mRNAs [fructose 1,6-biphosphate aldolase (Genbank accession number U73114) for the experiment of Fig. 3A and a CNS tyrosine kinase (A. G. M. Bulloch, unpublished data) for Fig. 3B].
22. Dedicated to the memory of Professor Håkan Persson (1952–1993), who was one of the most enthusiastic initiators of this project. We thank G. Hauser, R. van Elk, A.-S. Nilsson, E. van Kesteren, C. Popelier, and A. Ahlsen for technical support; L. Johanson and T. Laan for secretarial help; A. Vlamis and A. Holmgren for their generous assistance with the BIAcore; K. Dreisewerd and F. Hillenkamp for sharing expertise in MALDI-MS; and all members of the Molecular Neurobiology group at Vrije Universiteit Amsterdam for cheerful assistance in snail milking. Sp75 baculovirus and antisera were the generous gift of G. Weskamp and L. Reichardt. Supported by grants from the Swedish Medical Research Council (MRC), the European Neuroscience Program, the Canadian MRC, the Canadian Neuroscience Network, the Canadian National Science and Engineering Research Council, and a special equipment grant from the Netherlands Organization for Research. M.F. was supported by a long-term fellowship from the European Molecular Biology Organization and subsequently by the Swedish MRC. A.G.M.B., N.I.S., and W.C.W. were supported by the Alberta Heritage Foundation for Medical Research.

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T Cell Telomere Length in HIV-1 Infection: No Evidence for Increased CD4$^+$ T Cell Turnover

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Progression to acquired immunodeficiency syndrome (AIDS) has been related to exhaustion of the regenerative capacity of the immune system resulting from high T cell turnover. Analysis of telomeric terminal restriction fragment (TRF) length, a marker for cellular replicative history, showed that CD8$^+$ T cell TRF length decreased but CD4$^+$ T cell TRF length was stable during the course of human immunodeficiency virus type–1 (HIV-1) infection, which was not explained by differential telomerase activity. This observation provides evidence that turnover in the course of HIV-1 infection can be increased considerably in CD8$^+$ T cells, but not in CD4$^+$ T cells. These results are compatible with CD4$^+$ T cell decline in HIV-1 infection caused by interference with cell renewal.

In the course of HIV-1 infection, CD4$^+$ T cells are progressively lost, CD8$^+$ T cell numbers gradually increase, and immune function is progressively disturbed (*1*). Chronic immune activation is reflected by an activated phenotype of CD8$^+$ T cells in blood and lymph nodes (*2*), high concentrations of circulating HIV-specific cytotoxic T lymphocyte (CTL) effectors that are highly activated (*3*), and activation-induced programmed cell death that affects both CD8$^+$ and CD4$^+$ T cells (*4*). CD4$^+$ T cell numbers decline at an accelerated rate about 1.5 to 2 years before the onset of AIDS (*5*). It has been proposed that HIV-induced rapid CD4$^+$ T cell turnover eventually leads to exhaustion of the regenerative capacity of the immune system (*6*, *7*).

To study T cell turnover, we analyzed telomeric TRF length. Telomeres are the extreme ends of chromosomes that consist of TTAGGG repeats, ~10 kb long in humans (*8*). After each round of cell division telomeric sequence is lost (*9–12*) because of the inability of DNA polymerases to fully replicate the 5′ end of the chromosome (*13*). Cross-sectional studies have revealed a loss of 30 to 50 base pairs (bp) per year for human leucocytes in vivo (*9*, *10*, *14*), and telomere length has been used as a marker for replicative history and the proliferative potential of cells (*9–11*, *15*, *16*). To overcome the considerable variation in lymphocyte telomere length between donors of the same age (*17*), we analyzed TRF length on sequential peripheral blood mononuclear cell (PBMC) samples. For these analyses, the subtelomeric probe pTH2Δ (*18*) was chosen because it does not result in disproportionally high signals for longer telomeric

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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE RECOMBINANT HUMAN NERVE GROWTH FACTOR

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SUMMARY. The human NGF gene was isolated and inserted downstream from murine leukemia virus LTR in a plasmid having dihydrofolate reductase cDNA. The expression plasmid was introduced into CHO cells. Selection of the transformants for the resistance to methotrexate gave a CHO cell line which produced human NGF at a level of 4mg/L in the culture medium. The recombinant human NGF was purified to near homogeneity from the culture supernatant. The NH_2-terminal amino acid sequence, the COOH-terminal amino acid (Ala), and the amino acid composition of the human NGF were identical to those deduced from the nucleotide sequence of the human NGF gene. The recombinant human NGF was composed of 120 amino acid residues. Three disulfide likages were determined to be Cys^{15}-Cys^{80}, Cys^{58}-Cys^{108}, and Cys^{68}-Cys^{110}; the locations were identical to those in the mouse 2.5S NGF molecule. The specific biological activity of the recombinant human NGF was comparable with that of authentic mouse 2.5S NGF as determined by stimulation of neurite outgrowth from PC12 cells.

Nerve growth factor (NGF) is a protein essential for the development and maintenance of sympathetic and sensory neurons in the peripheral nervous system(1,2). Recent studies have shown that it may also be important for the development and maintenance of cholinergic neurons of the basal forebrain(3,4,5). NGF has been isolated from mouse submaxillary gland as β-subunit (β-NGF) which is composed of two identical polypeptides of 118 amino acid recidues.The amino acid sequence of β-NGF has been determined(6,7).

Little is known about human NGF (hNGF) presumably because of its low content in human tissues. The hNGF gene was cloned by Ullrich *et al.*(8). The amino acid sequence of hNGF deduced from the nucleotide sequence exhibits 90% similarity with that of mouse β-NGF(8). Although several papers have appeared so far describing the expression of the hNGF gene by *E.coli*, *S.cerevisiae* and COS cells(9,10,11), the productivity of these

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organisms and the specific biological activity of the products were extremely low. Therefore, the physicochemical properties of these recombinant hNGF proteins have remained unclear. No papers have described the production of biologically active hNGF in an amount enough to investigate its protein-chemical or biological nature in detail.

In this paper, we describe the production of recombinant hNGF by Chinese hamster ovary (CHO) cells and its purification and characterization.

MATERIALS AND METHODS

Materials: Mouse 2.5S NGF was purchased from Wako Pure Chemical Industries, Ltd.. CHO cells were obtained through Dr.P.Berg. PC12 cells were purchased from the American Type Culture Collection.

Construction of expression plasmid: The hNGF gene was isolated from the Lambda EMBL3 human leukocyte genomic library(Clontech) using a 0.38kb synthetic DNA encoding hNGF(8) as a probe. A positive clone having a 15kb insert was obtained. The nucleotide sequence of an open reading frame found in the insert was identical to that of the open reading frame encoding the prepro-NGF (from Met^{-121} to Ala^{120}) reported by Ullrich et al.(8). A 0.8kb BclI-ApaI fragment encoding the COOH-terminal half of the pre-peptide,pro-peptide and mature NGF was isolated from the insert and ligated with a synthetic DNA encoding the NH_2-terminal half of the pre-peptide. The resulting fragment containing the intact hNGF gene was inserted downstream from the murine leukemia virus(MuLV) LTR in the plasmid pTB399(12) ,from which the interleukin-2 cDNA had been removed, to give plasmid pTB1054. A 2kb ClaI fragment containing the MuLV LTR and hNGF gene was isolated from pTB1054 and inserted into the ClaI site in the plasmid pTB348(12) having the dihydrofolate reductase(DHFR) cDNA to give an expression plasmid pTB1058 (Fig.1).

Culture conditions: Transformed CHO cells were grown in Dulbecco's modified Eagle's medium(DMEM) containing 5% fetal calf serum,35μg/ml proline,50IU/ml penicillin,and 50μg/ml streptomycin at 37°C in 5% CO_2.

Purification of recombinant hNGF: (p-Amidinophenyl)methanesulfonyl fluoride hydrochloride was added to the culture supernatant at a final concentration of 0.1mM. The culture supernatant was adjusted to pH6.0 with 0.2N acetic acid and centrifuged. The supernatant was applied to a S-Sepharose column (2.5X15cm) equilibrated with 0.1M phosphate buffer(pH6.0)-1mM EDTA. The column was washed with 0.1M phosphate buffer(pH6.0)-1mM EDTA-10% glycerol-0.15M NaCl, and then the recombinant hNGF was eluted with 20mM Tris-HCl (pH7.4)-1mM EDTA-10% glycerol-0.7M NaCl. Fractions containing hNGF were

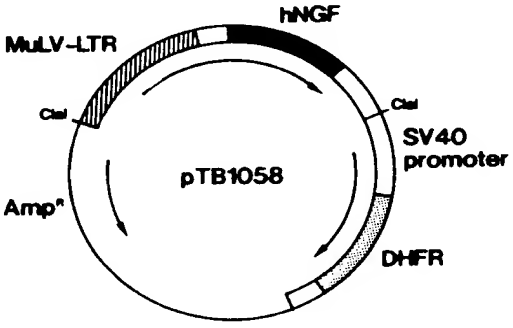

Fig.1. Structure of the expression plasmid for hNGF gene. Arrows indicate the direction of transcription.

combined and concentrated by ultrafiltration with an Amicon YM10 filter. The concentrate was applied to a Sephacryl S-100 HR (1.6X90cm) column equilibrated with 20mM Tris-HCl(pH7.4)-1mM EDTA-10% glycerol-0.7M NaCl, and the active entity was eluted with the same buffer. Fractions containing hNGF were combined and concentrated with Centriprep 10 (Amicon). The concentrate was subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on Asahipak ODP-50 (0.46X15cm) with a linear gradient of 0-90% acetonitrile containing 0.1% trifluoroacetic acid. The eluate containing hNGF was concentrated in vacuo to give purified hNGF as white powder.

Enzyme immunoassay(EIA): EIA was performed as described by Heinrich and Meyer(13).The purified hNGF obtained in this study was used as standard. The weight of the standard hNGF was determined by amino acid analysis.

Protein determination: Protein content was determined by BCA protein assay (Pierce) with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli(14).

Estimation of isoelectric point: The isoelectric point was estimated with an LKB 2117 multiphore II electrophoresis unit (Pharmacia-LKB) according to the directions of the supplier.

NH_2-terminal amino acid sequence, COOH-terminal amino acid, and amino acid composition: The NH_2-terminal amino acid sequence was determined with a gas phase protein sequencer (Model 470A,Applied Biosystems). The COOH-terminal amino acid was determined by hydrazinolysis(15). The amino acid composition was determined after hydrolysis for 24 hr with 5.7N HCl at 110°C in the presence of 4% thioglycolic acid. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer.

Determination of disulfide linkages: After digestion of purified hNGF with pepsin, the reaction mixture was loaded on a RP-HPLC column, TSK-gel ODS-120T (Tosoh)in the presence or absence of 20mM dithiothreitol, and the fragments were eluted by increasing acetonitrile concentration in the presence of trifluoroacetic acid. Peptide fragments containing disulfide linkages were determined by comparing the elution patterns. The fragments were further digested with thermolysin and the resulting smaller peptide frament were isolated by HPLC. The amino acid sequences and amino acid compositions of the cystine-containing peptides were determined.

Biological assay: Biological activity was assayed using PC12 cells as described by Stephani et al.(16) with some modifications.

RESULTS AND DISCUSSION

Establishment of the CHO cells producing hNGF

CHO $DHFR^-$ cells were transformed with pTB1058 by the calcium-phosphate coprecipitation method(17). $DHFR^+$ clones found to produce hNGF were then stepwise subcultured in the media containing 10nM, 100nM, 1μM, and 10μM methotrexate. A cell line, CHO-D31-10, obtained by this selection produced hNGF at a level of 4mg/L in the culture medium on the 7th day of growth.

Purification of recombinant hNGF

The cell line CHO-D31-10 was seeded at a density of $2.5X10^4$ cells/cm^2 and grown on a large scale. The recombinant hNGF was purified from the culture supernatant (2.2L) as described in MATERIALS AND METHODS. A summary of the purification is shown in Table 1. The amount of purified hNGF obtained was 1.6mg as determined by EIA. The value nearly accorded with that obtained from protein determination (1.3mg). SDS-polyacrylamide gel electrophoresis

Table 1. Summary of the purification of recombinant hNGF

Step	Volume (ml)	Total protein[1] (mg)	Total hNGF[2] (mg)	Yield (%)
Culture supernatant	2,200	11,000	5.3	100
S-Sepharose eluate	4.5	24	5.0	94
Sephacryl S-100 eluate	2.5	3.7	4.7	89
RP-HPLC eluate		1.3	1.6	30

1) Determined by BCA protein assay.
2) Determined by EIA.

(Fig.2) and RP-HPLC showed that the purity of the recombinant hNGF was more than 95%.

Properties of recombinant hNGF

The molecular weight of the recombinant hNGF was estimated to be 13,000 by SDS-polyacrylamide gel electrophoresis under reducing conditions. The hNGF migrated a little more slowly than did authentic mouse 2.5S NGF(Fig.2).

The isoelectric point of the recombinant hNGF was between 9 and 10; the mobility was nearly the same as that of the authentic mouse 2.5S NGF.

The NH_2-terminal amino acid sequence of the recombinant hNGF (NH_2-Ser-Ser-Ser-His-Pro-Ile-Phe-His-Arg-Gly-) was identical to that deduced from the nucleotide sequence of the hNGF gene(8). The COOH-terminal amino acid was determined to be Ala by hydrazinolysis, indicating that the recombinant hNGF has an additional two amino acid residues (Arg^{119}-Ala^{120}) at the COOH-terminus of the protein (118 amino acid residues) which Ullrich at al. proposed as mature hNGF based on the homology with mouse β-NGF (8). The amino acid composition of the purified hNGF was in fair agreement with that calculated from the deduced amino acid sequence consisting of 120 amino acid residues (Table 2).

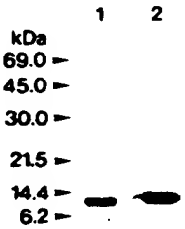

Fig.2. SDS-polyacrylamide gel electrophoresis under reducing conditions. Samples were (1) mouse 2.5S NGF and (2) recombinant hNGF(0.2 μg). Proteins were detected by silver staining. The sizes (kDa) of the standard proteins are shown on the left.

Table 2. Amino acid composition of hNGF

	Residues per molecule	
	Found	Calculated
Asx	13.0	13
Thr	9.7	10
Ser	9.2	11
Glx	6.6	6
Pro	2.9	3
Gly	7.3	7
Ala	6.9	7
Cys	ND	6
Val	12.8	13
Met	2.1	2
Ile	6.1	6
Leu	3.2	3
Tyr	2.3	2
Phe	7.3	7
Lys	9.1	9
His	3.9	4
Arg	7.3	8
Trp	3.0	3
Total		120

ND, Not determined.

On digestion with pepsin, a single peptide containing all the three disulfide linkages were isolated. On further digestion of this peptide with thermolysin, three fragments (fragments 1, 2, and 3) were obtained, each containing a disulfide linkage. The amino acid sequence of each fragment was determined. Three disulfide likages were found to be located at Cys^{15}-Cys^{80}, Cys^{58}-Cys^{108}, and Cys^{68}-Cys^{110} (Table 3). The presence of two cysteine residues in each fragment was confirmed by amino acid analysis employing the hydrolysis in thioglycolic acid (data not shown). The locations of the three disulside likages were identical to those in the

Table 3. Amino acid sequence of cystine-containing peptides

cycle	Fragment 1 residue (pmole)		Fragment 2 residue (pmole)		Fragment 3 residue (pmole)
1	Ser (60)		Phe (90)	Ala (86)	Val (115)
2	Val (43)	Tyr (43)	Glu (78)		Asp (70)
3	-	-	Thr (45)		Ser (8)
4	Asp (43)	Thr (28)	Lys (62)		Gly (47)
5	Ser (17)		-		-
6			Arg (78)		Arg (27)
7			Asp (49)		Gly (24)
8			Pro (33)		
9			Asn (31)		
10			Pro (11)		

S^{13}-V-C^{15}-D-S^{17}
S^{78}-Y-C^{80}-T^{81}

F^{54}-E-T-K-C^{58}-R-D-P-N-P^{63}
A^{107}-C^{108}

V^{64}-D-S-G-C^{68}-R-G^{70}
V^{109}-C^{110}

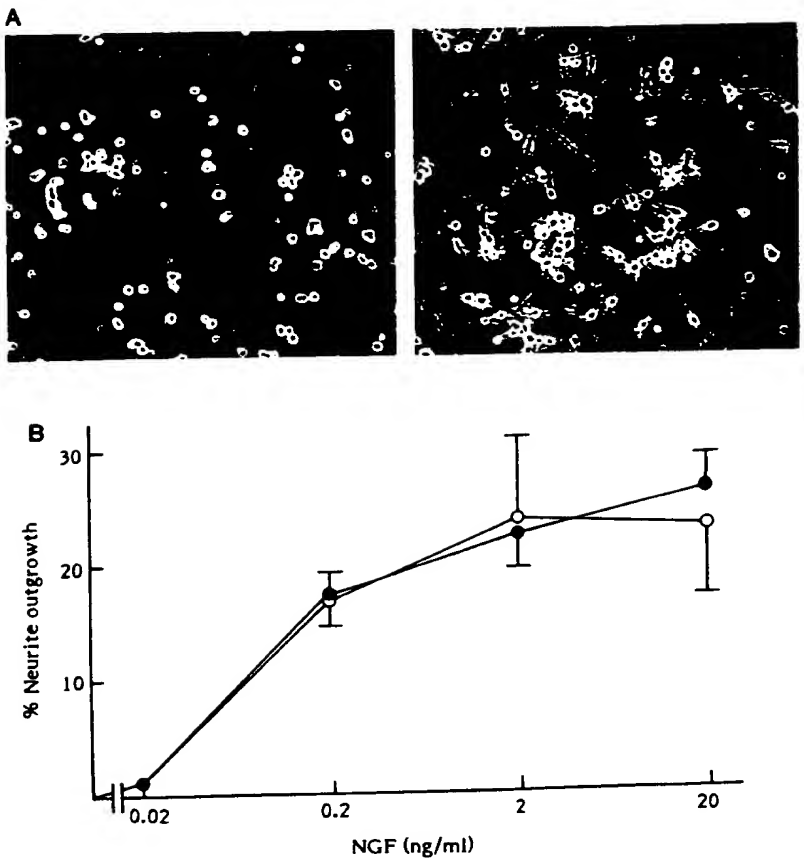

Fig.3. Biological activity of recombinant hNGF. (A) PC12 cells grown for 10 days in the absence(left) or presence(right) of hNGF(50ng/ml). (B) Dose-response curves for hNGF and mouse NGF. The primed PC12 cells were grown for 2 days in the presence of hNGF(closed circle) or mouse 2.5S NGF(open circle), and the numbers of cells with neurites were counted. The experiments were carried out in duplicate.

mouse NGF molecule (18,19), suggesting that the recombinant hNGF assumes the correct tertiary structure. The molecular weight of the recombinant hNGF was calculated to be 13,489.

Biological activity of recombinant hNGF

The recombinant hNGF stimulated neurite outgrowth from PC12 cells (Fig.3A). The activity was as high as that of the authentic mouse 2.5S NGF (Fig.3B). It also supported the survival of sensory neurons isolated from dorsal root ganglion of embryonic chicks (data not shown). The specific biological activity of recombinant mouse NGF and hNGF produced by E.coli (20) and S.cerevisiae (10),respectively, are reported to be 1/200-1/1000 of that of mouse NGF isolated from submaxillary gland(10,20). This may be due to incorrect folding of these recombinant proteins. In contrast, the

specific activity of the hNGF described here was comparable with that of authentic mouse 2.5S NGF. These results suggest that CHO cells produced the recombinant hNGF protein as a native form with a correct tertiary structure.

We have succeeded in producing substantial amounts of biologically active hNGF by the recombinant DNA technique using CHO cells. This will open the way for the basic and applied research on hNGF as a neurotrophic factor.

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NADE, a p75NTR-associated Cell Death Executor, Is Involved in Signal Transduction Mediated by the Common Neurotrophin Receptor p75NTR*

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The low affinity neurotrophin receptor p75NTR can mediate cell survival as well as cell death of neural cells by NGF and other neurotrophins. To elucidate p75NTR-mediated signal transduction, we screened p75NTR-associated proteins by a yeast two-hybrid system. We identified one positive clone and named NADE (p75NTR-associated cell death executor). Mouse NADE has marked homology to the human HGR74 protein. NADE specifically binds to the cell-death domain of p75NTR. Co-expression of NADE and p75NTR induced caspase-2 and caspase-3 activities and the fragmentation of nuclear DNA in 293T cells. However, in the absence of p75NTR, NADE failed to induce apoptosis, suggesting that NADE expression is necessary but insufficient for p75NTR-mediated apoptosis. Furthermore, p75NTR/NADE-induced cell death was dependent on NGF but not BDNF, NT-3, or NT-4/5, and the recruitment of NADE to p75NTR (intracellular domain) was dose-dependent. We obtained similar results from PC12 cells, nnr5 cells, and oligodendrocytes. Taken together, NADE is the first signaling adaptor molecule identified in the involvement of p75NTR-mediated apoptosis induced by NGF, and it may play an important role in the pathogenesis of neurogenetic diseases.

A member of the tumor necrosis factor receptor family, p75NTR, can medicate cell death under certain conditions (1–5). Unlike the Trk receptor family, its functional roles and signaling pathways have remained largely unclear (6). p75NTR (ICD)[1] has a C-terminal region that is highly homologous to a type-2 death domain, thus the existence of p75NTR (ICD)-binding proteins has been implicated (7). Recently, tumor necrosis factor receptor-associated factor (TRAF) family proteins, FAP-1, and zinc finger proteins have been reported to interact with p75NTR (ICD) (8–12), and these proteins potentially are involved in p75NTR-mediated signal transduction. However, none of them had a direct effect on NGF-dependent apoptosis. To further identify p75NTR (ICD)-binding proteins, we used rat p75NTR (ICD) as a target to screen mouse cDNA libraries by a yeast two-hybrid system. Here we report the identification of the p75NTR-associated cell death executor, NADE.

MATERIALS AND METHODS

Cloning of NADE—mNADE was isolated by using a yeast two-hybrid system as described previously (13); the cytosolic domain of p75NTR cDNA (amino acids 338–396) was used as a target. Briefly, we introduced mouse embryo pVP16 cDNA libraries into the LexA/p75NTR-expressing cells by using a high efficiency LiOAc transformation method (14). From a screen of 5×10^7 transformants, we identified an initial set of 672 His^+ colonies. Using a β-galactosidase colorimetric assay (15), we selected 181 clones as candidates for further analysis. One clone, which specifically reacted with the LexA/p75NTR, was selected and was named NADE. A full-length mNADE cDNA was cloned into pBluescript II KS(+) by inserting the partial NADE cDNA (nucleotides 7–524) and the 5′-rapid amplification of cDNA end product. PCR was used to replace the stop codon and to add 5′ *Xho*I and 3′ *Bam*HI sites into the full-length NADE cDNA. pcDNA3.1/myc-His(−)A/mNADE was constructed by inserting a full-length mNADE cDNA into *Xho*I-*Bam*HI-digested pcDNA3.1/myc-His(−)A (Invitrogen). Human NADE cDNA was amplified from a Jurkat T cell cDNA library; the product was then cloned into *Xho*I-*Bam*HI-digested pcDNA3.1/myc-His(−)A.

Plasmids and Analysis of Subcellular Localization—Expression plasmids for green fluorescence protein (GFP)-fused mNADE proteins were made as follows: GFP cDNA was PCR-amplified from pEGFP-N2 (CLONTECH) by using the primer pair 5′-CTAGCTAGCATCATGGTGAGCAAGGGCGAG-3′ and 5′-CCGCTCGAGTCTTGTACAGCTCGTCCAT-3′. The product was cloned into *Nhe*I-*Xho*I-digested pcDNA

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§ The first four authors contributed equally to this work.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF187064, AF187065, and AF187066.

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[1] The abbreviations used were: ICD, intracellular domain; BDNF, brain-derived neurotrophin factor; NGF, nerve growth factor; NT, neurotrophin; RT, reverse transcription; PCR, polymerase chain reaction; p75NTR, p75 neurotrophin receptor; PARP, poly(ADP-ribose) polymerase; GFP, green fluorescence protein; PBS, phosphate-buffered saline; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ALLM, *N*-acetyl-Leu-Leu-methioninal; PSI, *Z*-Ile-Glu(OtBu)-Ala-Leu-aldehyde; MG132, *Z*-Leu-Leu-Leu-aldehyde; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole.

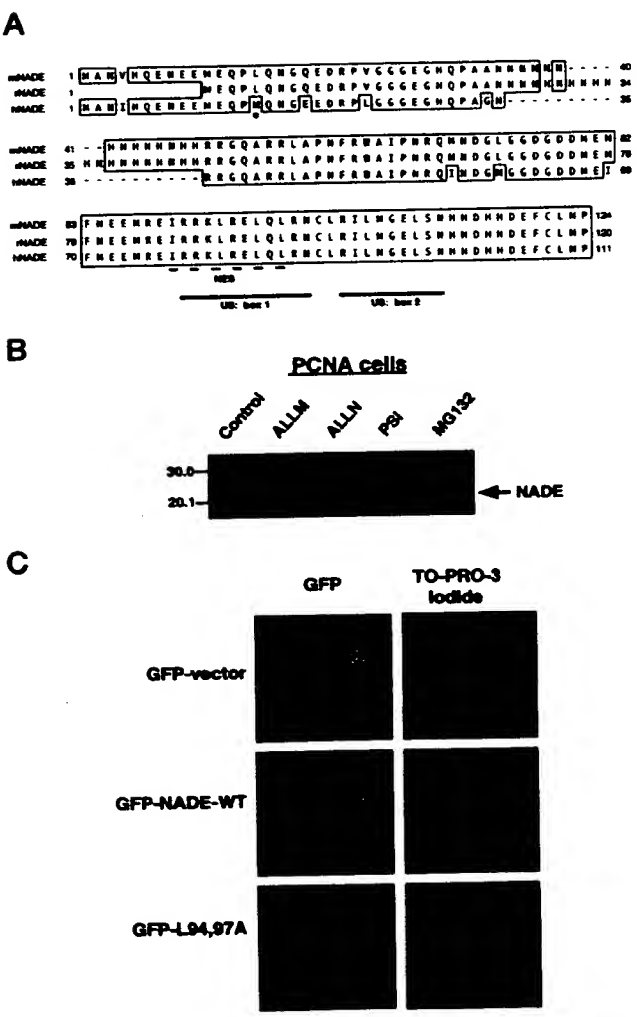

FIG. 1. **Sequence and expression of NADE.** *A*, amino acid alignment of mouse, rat, and human NADE (HGR74) (*18*) proteins. The *asterisk* indicates the alternative starting methionine in human NADE. The *dashed line* indicates the leucine-rich nuclear export signal (*NES*) (*19*). The *line* indicates the ubiquitination sequence (*US*) (20). GenBank™ accession numbers are as follows: mouse NADE, AF187066; rat NADE, AF187065; and human NADE (HGR74), AF187064, M38188. *B*, proteasome inhibitors induce expression of NADE proteins in PCNA cells. Cells were treated with the proteasome inhibitor ALLN (25 μM), PSI (10 μM), or MG132 (10 μM) or with a calpain inhibitor ALLM (25 μM) for 3 h. Cell lysates were subjected to 12.5% SDS-PAGE and analyzed by Western blotting with the anti-α-NADE polyclonal antibody. *C*, subcellular localization analysis of NADE protein in 293T cells. Cells were transfected with GFP, GFP-NADE wild type (*GFP-NADE-WT*), or GFP-NADE with L94A and L97A (GFP-L94, 97A), which have point mutations within NES motif. TO-PRO-3 iodide was used to visualize the nucleus, and the subcellular localization analysis was performed as described under "Materials and Methods."

3.1/*myc*-His(−)A/mNADE. We used the mutagenic primer pairs 5′-AAAGCTTAGGGAGGCACAGCTGAGAAA-3′ and 5′-TTTCTCAGCTGTGCCTCCCTAAGCTTT-3′ and 5′-ATCCGGAGAAAGGCTAGGGAGGCACA-3′ and 5′-TGTGCCTCCCTAGCCTTTCTCCGGAT-3′ to generate GFP-L94, 97A, in which both Leu-94 and Leu-97 are replaced with Ala. At 24 h after transfection, 293T cells transfected with GFP-containing plasmids were fixed with 3.7% paraformaldehyde, washed with PBS, and stained with TO-PRO-3 iodide to visualize the nucleus. The images of representative fields were captured on a Zeiss LSM 510 confocal laser-scanning microscope.

Reagents and Antibodies—*N*-acetyl-Leu-Leu-norleucinal (ALLN), *N*-acetyl-Leu-Leu-methioninal (ALLM), and mouse NGF were obtained from Sigma. PSI (*Z*-Ile-Glu-(OtBu)-Ala-Leu-aldehyde) and MG132 (*Z*-Leu-Leu-Leu-aldehyde) were obtained from Calbiochem. BDNF, NT-3, and NT-4/5 were obtained from PeproTech EC. TO-PRO-3 iodide was obtained from Molecular Probes. The anti-α-NADE polyclonal antibody was prepared by immunizing rabbits with the GST-mNADE fusion protein and was purified by using antigen-coupled Sepharose 4B (Amersham Pharmacia Biotech). The anti-rat p75NTR monoclonal antibody was obtained from Chemicon. The anti-caspase-3 monoclonal antibody and the anti-PARP polyclonal were obtained from Santa Cruz. The anti-mouse IgG-Cy-5 antibody and were purchased from Jackson Immunochemical. The anti-mouse Myc antibody, anti-FLAG antibody, and the anti-rabbit IgG-FITC conjugate were obtained from the Medical & Biological Laboratories. The anti-rat p75NTR antibody, the anti-caspase-2 polyclonal antibody, and the anti-O1 mouse monoclonal antibody were kind gifts from Drs. M. V. Chao, L. A. Greene, and S. Pfeiffer, respectively.

Cell Culture and Transfection—293T and PCNA cells were obtained from American Type Culture Collection; PC12 and nnr5 cells were obtained from Dr. L. A. Greene. Oligodendrocytes were isolated as described previously (16). 293T and PCNA cells were maintained in DMEM supplemented with 10% fetal bovine serum. PC12 and nnr5 cells were maintained in RPMI 1640 supplemented with 10% horse serum and 5% calf serum. 293T cells (1.5×10^6/per 100-mm dish) were transiently transfected with 20 μg of plasmid according to the calcium phosphate method in DMEM supplemented with 10% fetal bovine serum. PC12 and nnr5 cells (4×10^6 per 100-mm dish) were transiently transfected with 12 μg of plasmid by using LipofectAMINE PLUS (Life Technologies, Inc.) in serum-free DMEM containing 100 ng/ml NGF.

Co-immunoprecipitation—The transfected 293T cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 7 μg/ml pepstatin, and 2 μM PSI) at 4 °C for 15 min, then the lysates were centrifuged at 15,000 × *g* for 30 min. The supernatants were immunoprecipitated at 4 °C for 8 h by using 1 mg of anti-Myc antibody coupled to protein G-Sepharose 4B. The beads were washed with lysis buffer, and the proteins were separated by 12.5% SDS-PAGE and analyzed by Western blotting using the anti-p75NTR polyclonal antibody or the anti-α-NADE polyclonal antibody. For PC12 cells, cells were treated with 100 ng/ml NGF in serum-free DMEM, lysed in 1 ml of lysis buffer at 4 °C for 15 min, and centrifuged at 15,000 × *g* for 20 min. The supernatants were immunoprecipitated by using 0.8 mg of anti-rat p75NTR monoclonal antibody (Chemicon) or anti-α-NADE polyclonal antibody, which were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The immunoprecipitated proteins were analyzed by Western blotting using the anti-rat p75NTR polyclonal antibody or the anti-α-NADE polyclonal antibody.

RT-PCR—TRIZOL Reagent (Life Technologies, Inc.) was used to isolate 5 μg of total RNA from oligodendrocytes after treatment of 10 to 100 ng/ml NGF for 3 h. NADE mRNA was amplified by using the primer pair 5′-CATTCCCAACAGGCAGATG-3′ and 5′-GGCATAAGGCAGAATTCATC-3′. β-Actin was used as a control.

Apoptosis Assays and Caspase Assays—The transfected cells were washed with PBS, fixed in 3.7% paraformaldehyde, and stained with 50 μg/ml DAPI. By using a fluorescence microscopy, the number of cells that had the nuclear morphology typical of apoptosis among at least 400 cells were counted in each sample. The MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories) was used for TUNEL assay (17) according to the manufacturer's protocol. Western blotting analysis detected the active forms of caspase-2, caspase-3, and PARP. For PC12 cells and nnr5, the caspase-3 assay was performed with a CPP32/caspase-3 Fluorometric Protease Assay Kit (Medical & Biological Laboratories) according to the manufacturer's instructions.

Immunocytochemistry—Oligodendrocytes were treated with 100 ng/ml NGF for 12 h. After incubation with the anti-O1 mouse monoclonal antibody, cells were fixed with 3.7% paraformaldehyde for 60 min at room temperature, permeabilized with 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min on ice, and incubated with the anti-α-NADE antibody in PBS containing 0.5 M NaCl, 1% bovine serum albumin, and 1% normal goat serum (Sigma) at room temperature for 1 h. After several rinses with PBS, cells were incubated with the anti-rabbit IgG-FITC conjugate (Medical & Biological Laboratories) and anti-mouse IgG-Cy-5 conjugate in PBS at room temperature for 30 min. For the TUNEL assay, after incubation with the anti-O1 monoclonal antibody, cells were fixed, permeabilized, and processed by using the *in situ* detection kit for apoptosis according to the manufacturer's instructions (Medical & Biological Laboratory). After several rinses with PBS, cells were incubated with the anti-mouse IgG-Cy-5 conjugate in PBS at room temperature for 30 min. Glass coverslips were mounted in glycerin jelly, and the images of representative fields were captured on a Zeiss LSM 510 confocal laser-scanning microscope.

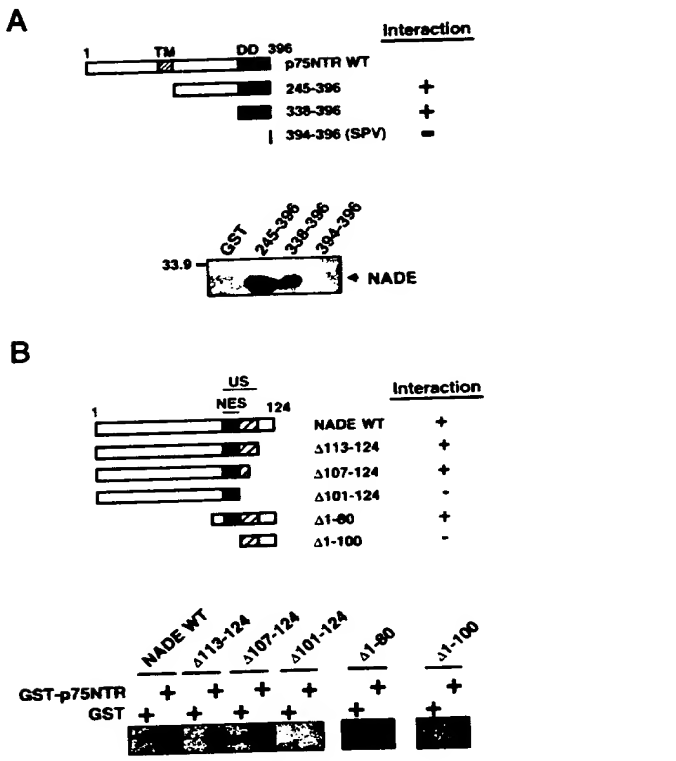

FIG. 2. **Interaction of NADE with p75NTR.** A, *top panel*: determination of the mNADE-binding domain in p75NTR *in vitro*. *TM*, transmembrane domain; *DD*, cytoplasmic death domain. *Bottom panel*, the interaction of mNADE with the cell death domain of p75NTR *in vitro*. GST-p75NTR fusion proteins and *in vitro* translated mNADE with [^{35}S]methionine were incubated and bound complexes were precipitated as described previously (12). *B*, *top panel*: determination of the p75NTR-binding domain in mNADE *in vitro*. The mNADE domains tested in the GST fusion proteins are shown schematically. *NES*, nuclear export signal; *US*, ubiquitination signal. *Bottom panel*, the interaction of mNADE with p75NTR was measured by using the GST fusion proteins containing the p75NTR cytoplasmic region (338–396) with either mNADE or its deletion mutants. *C*, mNADE associates with p75NTR in a ligand-dependent manner. Cells were transfected with pcDNA3/rat-p75NTR and pcDNA3.1/*myc*-His(−)A/mNADE and cultured for 10 h. After withdrawing the serum, cells were treated with 10 or 100 ng/ml NGF for 12 h. Coimmunoprecipitation was performed as described under "Materials and Methods." Expression of p75NTR and mNADE was confirmed by Western blotting using the anti-rat p75NTR and anti-mouse Myc antibodies. Anti-FLAG antibody was used as a control. *D*, native complex formation between NADE and p75NTR in PC12 cells as detected by co-immunoprecipitation assays. The anti-rabbit and the anti-mouse IgG antibodies were used as controls.

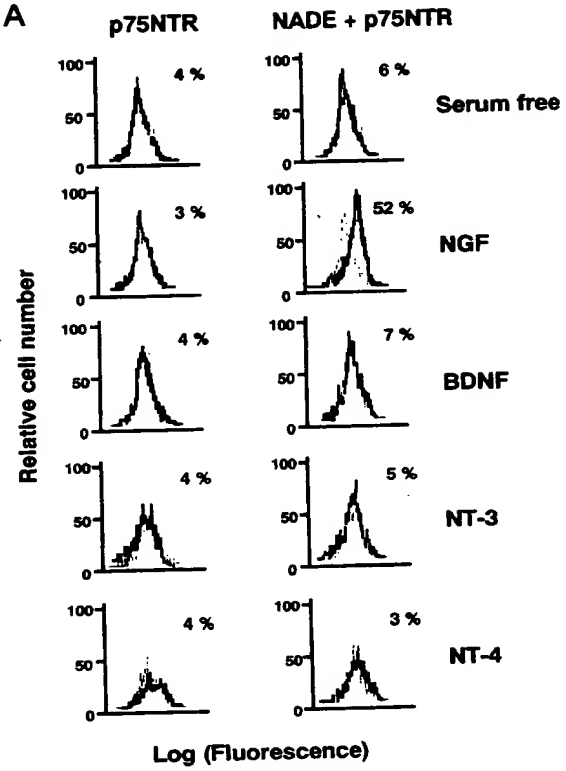

FIG. 3. **NGF-dependent regulation of p75NTR/NADE-induced apoptosis in 293T cells.** *A*, effect of neurotrophins on cell death initiated by the association of mNADE with p75NTR. Cells were transfected with both of an expression vector pcDNA vector pcDNA3.1/*myc*-His(−)A and pcDNA3/rat-p75NTR or both of pcDNA3/rat-p75NTR and pcDNA3.1/*myc*-His(−)A/mNADE and cultured for 10 h. After withdrawing the serum, the cells were treated with 100 ng/ml NGF BDNF, NT-3, or NT-4/5 for 36 h. The TUNEL assay was performed as described under "Materials and Methods." These representative histograms show the relative numbers of apoptotic cells. *B*, caspase activation induced by NGF-dependent p75NTR/NADE-mediated apoptosis. After serum depletion, cells were treated with 100 ng/ml NGF for 24 h. The activation of caspase-2 and caspase-3 was detected using anti-caspase-2 polyclonal and anti-caspase-3 monoclonal antibodies. The degradation of PARP was detected with the anti-PARP polyclonal antibody.

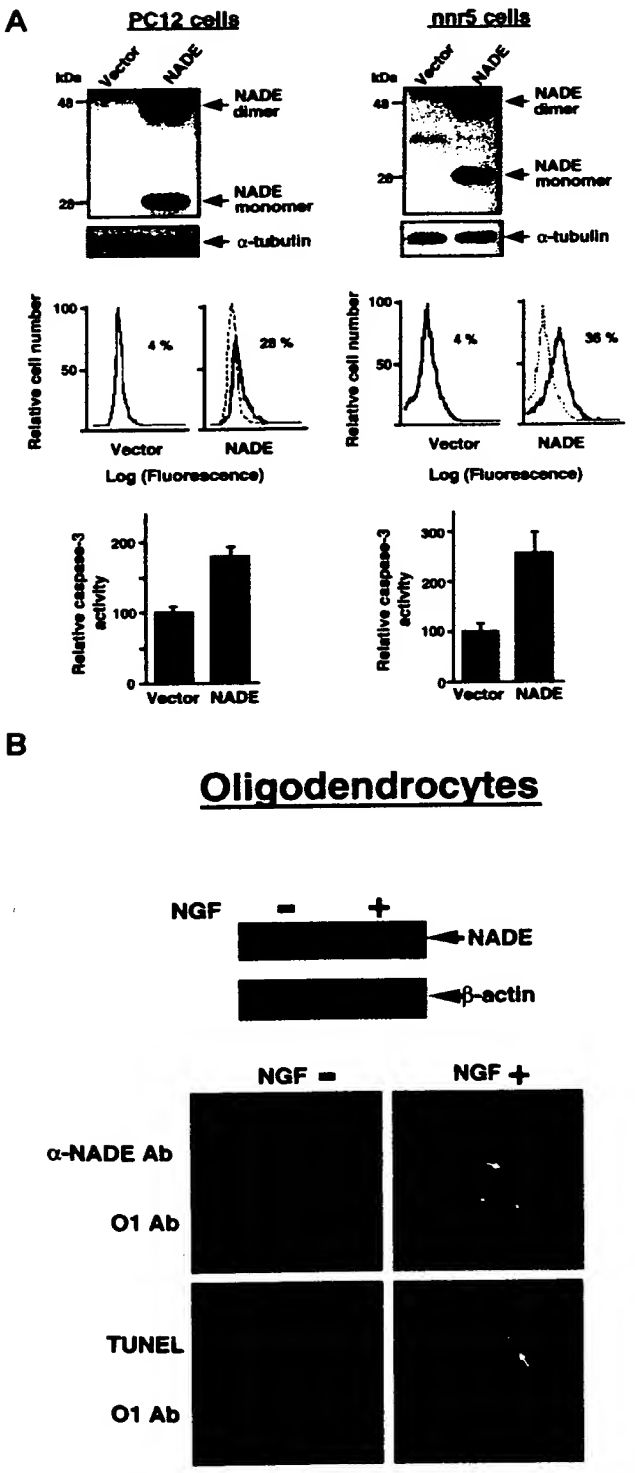

FIG. 4. **NGF-dependent regulation of NADE in PC12 cells, nnr5 cells, and oligodendrocytes.** *A*, transiently transfected mNADE induces apoptosis in PC12 and nnr5 cells. Cells were transfected for 48 h with pcDNA3.1/*myc*-His(−)A or pcDNA3.1/*myc*-His(−)A/mNADE in serum-free DMEM containing 100 ng/ml NGF. *Top panel*, expression of mNADE was detected by Western blotting with the anti-α-NADE polyclonal antibody. α-Tubulin was used as a control. *Middle panel*, the percentage of apoptotic cells was determined by the TUNEL assay, and these representative histograms show the relative number of apoptotic cells. *Bottom panel*, caspase-3 activation was detected as described under "Materials and Methods." *B*, NGF-dependent expression of NADE and its caspase activation in oligodendrocytes. *Top panel*, NGF-dependent expression of NADE mRNA was determined by RT-PCR.

RESULTS AND DISCUSSION

Mouse NADE (mNADE) comprises 124 amino acids, with a predicted molecular mass of 14,532 Da; this factor seems to be a hydrophilic and acidic protein (estimated pI = 5.97). Previously uncharacterized human gene HGR74 (18) showed significant identity to mNADE (92.8%, except the histidine-asparagine-rich stretch (amino acid residues 36–48; Fig. 1*A*)), being an obvious human homolog of NADE. NADE proteins in mouse, rat, and human have two consensus motifs, the leucine-rich nuclear export signal (NES) (19) and two boxes for ubiquitination sequences (20) (Fig. 1*A*). Expression of mNADE mRNA (1.3 kilobases) was found highest in brain, heart, and lung, with lesser amounts in stomach, small intestine, and muscle and no expression in liver (data not shown). Interestingly, NADE protein was detected in PCNA and PC12 cells only after treatment with proteasome inhibitors ALLN, PSI, and MG132 for 3 h (Fig. 1*B* and data not shown). These data imply that native NADE is modified by the ubiquitin conjugating system, thereby leading to subsequent protein degradation by proteasome. To investigate whether the NES sequence (19) in NADE is used for protein transportation from nucleus to cytosol, subcellular localization analyses of GFP-NADE and GFP-NADE NES mutants (Leu-94 → Ala and Leu-97 → Ala) were performed in 293T cells. Wild type with intact NES localizes to cytoplasm (Fig. 1*C*, *middle left panel*), but the NES mutants remain in nucleus (Fig. 1*C*, *bottom left panel*). These data suggested that NADE protein can be exported from nucleus to cytosol and that a NADE associating protein may promote and regulate the functional interaction of NADE with p75NTR.

To assess the interaction between mNADE and p75NTR, we first performed *in vitro* binding assays using GST fusion proteins (Fig. 2*A and B*). The mapping studies demonstrated that mNADE strongly binds to the cell death domain of p75NTR (ICD) (amino acids 338–396) which is identical among mouse, rat, and human sequence (Fig. 2*A*). In addition, C-terminal portion of NADE (amino acids 81–106) is necessary for the interaction with p75NTR (ICD) (Fig. 2*B*). To confirm *in vivo* interaction of NADE with p75NTR and its ligand dependence, we carried out co-immunoprecipitation experiments with *myc*-tagged mNADE/p75NTR co-transfected 293T cells. The results clearly showed that the recruitment of mNADE to p75NTR (ICD) was increased dose-dependently by NGF (Fig. 2*C*). In addition, the co-immunoprecipitation assays under physiological conditions in rat pheochromocytoma PC12 and the human neuroblastoma SK-N-MC cell lines confirmed the interaction of NADE with p75NTR (Fig. 2*D* and data not shown). Interestingly, two NADE bands (22 and 44 kDa) were detected in SDS-PAGE under reducing conditions.

To investigate the functional role of mNADE, we tested ligand-dependent apoptosis by the interaction of mNADE with p75NTR in 293T cells. Using TUNEL assay, we detected ligand-dependent cell death in the presence of NGF, but not BDNF, NT-3, or NT-4/5 in co-transfected 293T cells (Fig. 3*A*). In addition, NGF-treated 293T and COS7 cells transfected with mNADE and p75NTR showed morphological changes and DNA fragmentation typical for apoptosis (data not shown). These results strongly suggest that NADE has an important role in NGF-induced cell death by transducing the signal downstream

β-Actin was used as a control. *Bottom panels*, immunocytochemistry was performed as described under "Materials and Methods." *Red* indicates mature oligodendrocytes detected by the anti-O1 mouse monoclonal antibody and the anti-mouse IgG Cy-5-coupled secondary antibody. *Green* indicates NADE protein detected by using the anti-α-NADE antibody or the TUNEL assay with the FITC-dUTP. The superimposed *arrows* indicate positive staining of NADE protein and TUNEL.

of p75NTR. Next, we evaluated NGF-dependent caspase activation in transfected 293T cells. By Western blotting analysis, we showed that caspase-2 and caspase-3 were processed to active forms only in 293T cells that were co-transfected with mNADE and p75NTR and treated with 100 ng/ml NGF (Fig. 3*B*). Also under the same conditions, PARP was degraded (Fig. 3*B*). These data suggest that caspase-2 and caspase-3 are involved in NGF-induced cell death by p75NTR/NADE signal transduction pathway.

We used PC12 cells ($p75NTR^{+++}$, $TrkA^{+}$) (21, *22*), PC12 nnr5 cells ($p75NTR^{++}$, $TrkA^{-}$; these cells lack the TrkA receptor) (23) and mature oligodendrocytes ($p75NTR^{+++}$, $TrkA^{-}$) (24) to further study the physiological functions of NADE. Transiently transfected PC12 and nnr5 cells expressed NADE protein in the presence of NGF (Fig. 4*A*, *top panel*). Both TUNEL assay and DAPI staining clearly showed that the transient transfection of mNADE to PC12 and nnr5 cells caused their NGF-dependent cell death (Fig. 4*A*, *middle panel* and data not shown). In addition, caspase-3 was activated in both cell types (Fig. 4*A*, *bottom panel*), suggesting that TrkA receptor does not markedly modulate p75NTR/NADE-mediated apoptosis.

RT-PCR was used to examine the NGF-dependent induction of NADE expression in mature oligodendrocytes. The results clearly showed that NADE mRNA is induced in the presence but not in the absence of NGF (Fig. 4*B*, *top panel*). Furthermore, NGF induced caspase-3 activation (Ref. 16 and data not shown), implying a role for NADE in NGF-dependent p75NTR-mediated apoptosis (24). To further characterize the NGF-dependent cell death of mature oligodendrocytes, confocal laser-scanning microscopy was used to detect the cells undergoing apoptosis. In NGF-treated cultures, 60% of mature oligodendrocytes were TUNEL-positive, but in control cultures, only 10% or less were TUNEL-positive. In addition, 70% of mature oligodendrocytes were stained by NADE antibodies in NGF-treated cultures, compared with 5% or less in control cultures. After NGF treatment for 12 h, both nuclear TUNEL-positive staining and NADE-positive staining could be observed in more than 50% of O1-positive mature oligodendrocytes by immunocytochemistry (Fig. 4*B*, *bottom panels* and data not shown).

The signal cascade mediated by p75NTR has been enigmatic for a long time. Recent evidence suggest that p75NTR bifunctionally mediates signals to induce as well as inhibit apoptosis (25, 26). Our results strongly support the hypothesis that NADE is a putative signal transducer for p75NTR-mediated apoptosis. Furthermore, co-induction of p75NTR and NADE triggers zinc-induced neuronal cell death in the model of rat hippocampus following ischemia.[2] Importantly, since NADE contains NES as well as ubiquitination sequences, its protein levels and localization might be under tight control in normal cells. Taken together, our data showed that the functional interaction of NADE with p75NTR plays an important role in p75NTR-mediated signal transduction under physiological conditions.

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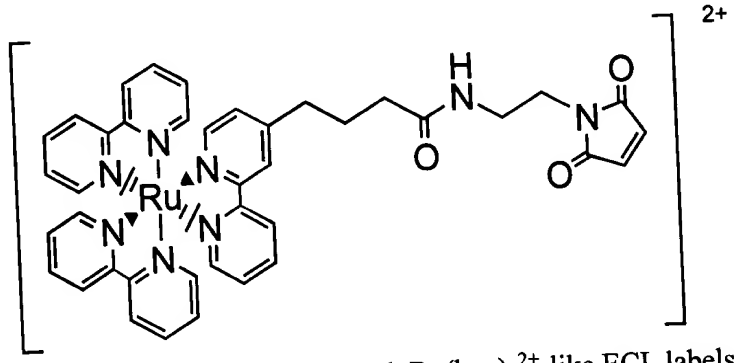
reacting an active ester derivative, such as that shown above, with a maleidmido-alkylamine (*e.g.*, maleimidoethylamine). Such a compound is 4-(maleimido-ethylamino-carbonylpropyl)-4'-methyl-2,2'-bipyridine bis(2,2'-bipyridine) ruthenium(II) dihexafluorophosphate, the cation of which is shown below. This maleimide permits the easy attachment of the $Ru(bpy)_3^{2+}$-like ECL label to molecules which possess, for example, a thiol group (*e.g.*, -SH).



Other examples of metal chelates from which $Ru(bpy)_3^{2+}$-like ECL labels may be derived include:

bis[(4,4'-carbomethoxy)-2,2'-bipyridine]-2-[3-(4-methyl-2,2'-bipyridine-4'-yl) propyl]-1,3-dioxolane ruthenium (II);

bis(2,2'-bipyridine)-[4-(butan-1-al)-4'-methyl-2,2'-bipyridine] ruthenium (II);

bis(2,2'-bipyridine)-[4-(4'-methyl-2,2'-bipyridine-4'-yl)-butyric acid] ruthenium (II);

bis(2,2'-bipyridine)-[4-(4'-methyl-2,2'-bipyridine)-butylamine] ruthenium (II);

bis(2,2'-bipyridine)-[1-bromo-4(4'-methyl-2,2'-bipyridine-4'-yl)butane] ruthenium (II); and

bis[(2,2'-bipyridine)maleimidohexanoic acid]-4-methyl-2,2'-bipyridine-4'-butylamide ruthenium (II).

Other examples of metal chelates from which ECL labels may be derived include other 2,2'-bipyridyl complexes, such as $Os(bpy)_3^{2+}$ and derivatives thereof; phenanthroline (phen) and derivatives thereof; other transition metal fluorophores, such as tricarbonyl(chloro)(1,10-phenanthroline) rhenium(I), square planer platinum(II) complexes, $Cr(bpy)_3^{2+}$; multinuclear complexes such as $Pt_2(diphosphonate)_4^{4-}$; and clusters such as $Mo_6Cl_{12}^{2-}$.

Another important class of chemical moieties which may serve as an ECL label are those derived from polyaromatic hydrocarbons, such as naphthalene, anthracene, 9,10-diphenylanthracene, phenanthrene, pyrene, chrysene, perylene, coronene, rubrene, and the like, and from organic laser dyes, such as fluorosceine, rhodamine, and the like, which are able to emit light upon electrochemical excitation.

Typically, one or more ECL labels are attached (*e.g.*, conjugated) to another molecule (*e.g.*, an antibody, an oligonucleotide probe). ECL labels may be attached to molecules (to form labeled molecules) using standard synthetic methods which are well known to one of skill in the art. For example, as discussed above, a molecule comprising an ECL label (*e.g.*, $Ru(bpy)_3^{2+}$ or a derivative thereof) may be derivatized to form a chemically activated species (*e.g.*, an activated ester, a maleimide) which may then be reacted with, and thus covalently bound to, a molecule (*e.g.*, to yield a labeled assay reagent).

B. Quenching Moieties

As described above, ECL is the emission of photons of electromagnetic radiation (*e.g.*, light) from an electronically excited chemical species which has been generated, either directly or indirectly, electrochemically. The observed ECL emission may be partially or completed attenuated by a quenching moiety which is in quenching contact with an ECL label. The terms "quenching moiety" and "quencher" as used herein, pertain to a chemical moiety which, when in quenching contact with an ECL label, attenuates the observed ECL emission.

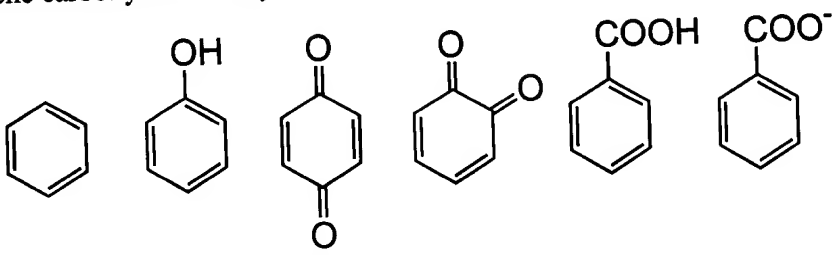
The phrase "in quenching contact with," as used herein, pertains to the condition wherein the observed ECL emission from an ECL label is attenuated by the presence of an ECL quenching moiety. A quenching moiety in quenching contact with an ECL label attenuates the observed ECL emission from that label by at least 10%. Preferably, a quenching moiety in quenching contact with an ECL label attenuates the observed ECL emission from that label by at least 20%, more preferably by at least 30%, still more preferably by at least 40%, yet more preferably by at least 50%. Typically, a quenching moiety in

quenching contact with an ECL label is physically present in spatial proximity to the ECL label. For example, a quenching moiety in quenching contact with an ECL label is typically separated from an ECL label by a distance of less than about 100 nm, more typically less than about 50 nm, still more typically less than about 30 nm, yet more typically less than about 10 nm. Using well known and standard methods, one of skill in the art may readily determine whether a prospective quenching moiety will in fact attenuate the observed ECL emission and also whether or not a specified quenching moiety is, in fact, in quenching contact with an ECL label.

Without wishing to be bound to any particular theory, Applicants note that a number of possible mechanisms for the quenching effect have been postulated. In one mechanism, the electronically excited label relaxes by transferring an electron to the quencher (perhaps by quantum mechanical tunneling), to yield an electronically excited quencher, which relaxes non-radiatively (*e.g.*, vibrationally, rotationally). In another mechanism, the electronically excited label relaxes by emitting a photon which is absorbed by the quencher, to yield an electronically excited quencher, which again relaxes non-radiatively. In still another mechanism, the quenching moiety is electrochemically converted to an electro-oxidation or electro-reduction product (typically during the ECL measurement), and this product (or subsequent reaction product) quenches the ECL, for example, by one of the preceding mechanisms. In yet another mechanism, the quenching moiety, or an electro-oxidation or electro-reduction product of the quenching moiety (or subsequent reaction product) acts as a free radical scavenger and intercepts one or more species involved in the ECL reaction sequence (*e.g.*, TPA$^{\bullet}$ may be intercepted prior to reaction with $Ru(bpy)_3^{3+}$, preventing the formation of $Ru(bpy)_3^{2+*}$) and thus quenching the ECL.

A number of chemical moieties may serve as a quenching moiety. An important class of such quenching moieties are those which comprise at least one benzene moiety. A sub-class of preferred quenching moieties are those which comprise at least one phenol moiety. Another sub-class of preferred quenching moieties are those which comprise at least one quinone moiety (*i.e.*, a 1,4-benzoquinone or a 1,2-benzoquinone). Yet another sub-class

of preferred quenching moieties are those which comprise at least one benzene carboxylic acid or benzene carboxylate moiety.



The term "quenching agent," as used herein, pertains to a chemical compound which comprises a quenching moiety. Examples of quenching agents which comprise at least one phenol moiety, and from which quenching moieties comprising at least one phenol moiety may be derived, include, but are not limited to:

phenol;

alkyl-phenols, such as C_{1-6} alkyl-phenols including *o*-alkyl-phenol, *m*-alkyl-phenol, and *p*-alkyl-phenol, such as *o*-methyl-phenol (*i.e.*, *o*-cresol), *m*-methyl-phenol (*i.e.*, *m*-cresol), *p*-methyl-phenol (*i.e.*, *o*-cresol), *o*-ethyl-phenol, *m*-ethyl-phenol, *p*-ethyl-phenol, *o*-propyl-phenol, *m*-propyl-phenol, and *p*-propyl-phenol;

aryl-phenols, such as C_{7-10} aryl-phenols, including *o*-aryl-phenol, *m*-aryl-phenol, and *p*-aryl-phenol, such as *p*-phenyl-phenol;

halo-phenols, including *o*-halo-phenol, *m*-halo-phenol, and *p*-halo-phenol, such as *o*-fluoro-phenol, *m*-fluoro-phenol, and *p*-fluoro-phenol;

hydroxy-phenols, including *o*-hydroxy-phenol (*i.e.*, catechol), *m*-hydroxy-phenol (*i.e.*, resorcinol), and *p*-hydroxy-phenol (*i.e.*, hydroxyquinone); and

biphenols, such as 4,4'-biphenol.

Examples of quenching agents which comprise at least one quinone moiety, and from which quenching moieties comprising at least one quinone moiety may be derived, include, but are not limited to:

quinones (*i.e.*, benzoquinones), such as *o*-quinone (*i.e.*, 1,2-benzoquinone) and *p*-quinone (*i.e.*, 1,4-benzoquinone);

alkyl-quinones, such as C_{1-6} alkyl-quinones including C_{1-6} alkyl-1,4-benzoquinones, such as 2-methyl-1,4-benzoquinone, 2-ethyl-1,4-benzoquinone, 2-*n*-propyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, and 2,5-dimethyl-1,4-benzoquinone;

halo-quinones, such as halo-1,4-benzoquinones, including 2-fluoro-1,4-benzoquinone, 2-chloro-1,4-benzoquinone, 2-bromo-1,4-benzoquinone, 2-iodo-1,4-benzoquinone, 2,6-difluoro-1,4-benzoquinone, 2,5-difluoro-1,4-benzoquinone; 2,6-dichloro-1,4-benzoquinone, 2,5-dichloro-1,4-benzoquinone; 2,6-dibromo-1,4-benzoquinone, and 2,5-dibromo-1,4-benzoquinone;

naphthoquinones, such as 1,2-napththoquinones and 1,4-naphthoquinones, including 2-methoxy-3-methyl-1,4-naphthoquinone;

anthraquinones, such as 1,2-anthraquinones, 1,4-anthraquinones, 9,10-anthraquinones, including 1,5-dihydroxy-9,10-anthraquinone, 1,2,3,4-tetrafluoro-5,8-dihydroxy-9,10-anthraquinone, 9,10-anthraquinone-2-carboxylic acid, 9,10-anthraquinone-2-sulfonic acid, 9,10-anthraquinone-1,5-disulfonic acid, and 9,10-anthraquinone-2,6-disulfonic acid.

Examples of quenching agents which comprise at least one benzene carboxylic acid or benzene carboxylate moiety, and from which quenching moieties comprising at least one benzene carboxylic acid or benzene carboxylate moiety may be derived, include, but are not limited to:

benzoic acid;

aminobenzoic acids, such as *o*-aminophenol, *m*-aminophenol, and *p*-aminophenol;

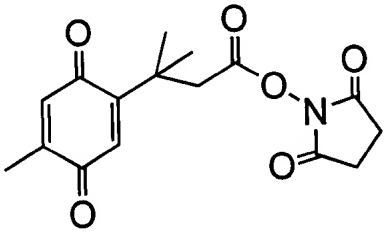
hydroxybenzoic acids, such as *o*-hydroxyphenol, *m*-hydroxyphenol, and *p*-hydroxyphenol; and

nitrobenzoic acids, such as *o*-nitrophenol, *m*-nitrophenol, and *p*-nitrophenol.

In one embodiment, the quenching moiety is a quinone or a derivative thereof. Quinone and its derivatives may usually be chemically modified to possess reactive groups (*i.e.*, to form chemically activated species). For example, on one or more reactive groups may be attached (*e.g.*, at the *ortho*- or *meta*-positions of 1,4-benzoquinone) optionally *via* a linker

group, which then permits the attachment of the quinone-like moiety (as a quenching moiety) to other molecules.

For example, a 1,4-benzoquinone may be derivatized to possess a carboxylic acid group (*i.e.*, -COOH) attached to an *ortho*- or *meta*-carbon *via* a linker group, such as an alkyl group. Such a compound is 2-(1-carboxy-but-2-yl)-5-methyl-1,4-benzoquinone. This carboxylic acid derivative may be derivatized to form the N-succinimidyl ester (shown below), which permits the easy attachment of the quinone-like quenching moiety to molecules which possess, for example, an amino group.



Quenching moieties may be attached to molecules using standard and well known synthetic methods. For example, as discussed above, a molecule comprising a quenching moiety (*e.g.*, benzene or a derivative, such as phenol, quinone, benzene carboxylic acid, benzene carboxylate) may be derivatized to form a chemically activated species (*e.g.*, an active ester, a maleimide) which may then be reacted with, and thus covalently bound to, a molecule.

C. Assays Employing an ECL Label and an ECL Quencher

The present invention provides new assay methods for detecting, and preferably quantifying, one or more analytes of interest which are present in a sample composition. The terms "assay" and "assay method," as used herein, pertain to a method of detecting the presence of (*e.g.*, qualitative assay), and preferably quantifying (*e.g.*, quantitative assays), one or more analytes of interest.

Assays of the present invention generally involve contacting the analyte of interest (which is typically one component of a sample composition) with a pre-determined non-limiting amount of one or more assay reagents, measuring the ECL properties of a

resulting product (the detection product(s)), and correlating the measured ECL with the amount of analyte present in the original sample, typically by using a relationship determined from standard samples containing known amounts of analyte of interest in the range expected for the sample to be tested. In a qualitative assay, simply determining whether the measured ECL is above or below a threshold value (established, for example, using samples known to contain or be free of analyte of interest) may be sufficient to establish the assay result. Thus, unless otherwise required, the term "measuring" can refer to either qualitative or quantitative determination. Assays of the present invention may be heterogeneous (separation) assays or homogeneous (non-separation) assays.

The terms "analyte" and "analyte of interest," as used herein, pertain to a substance which is to be detected and preferably quantified. Analytes may be inorganic or organic, though typically they are organic. Analytes may be naturally occurring or synthetic. Examples of classes of organic analytes include biological molecules such as amino acids, proteins, glycoproteins, lipoproteins, saccharides, polysaccharides, lipopolysaccharides, fatty acids, and nucleic acids. Examples of organic analytes include antibodies, antigens, haptens, enzymes, hormones, steroids, vitamins, oligonucleotides, and pharmacological agents.

The terms "sample" and "sample composition," as used herein, pertain to a composition which comprises one or more analytes of interest, or which may be processed to comprise one or more analytes of interest. The sample may be in solid, emulsion, suspension, liquid, or gas form. Typically, the sample is processed (*e.g.*, by the addition of a liquid electrolyte) so as to be a fluid (*i.e.*, free flowing) form (*e.g.*, emulsion, suspension, solution) in order to readily permit and simplify the detection and quantification of the analytes of interest using ECL methods. Typically, the analyte of interest is present in the sample composition at a concentration of 10^{-3} M (micromolar) or less, for example, often as low as 10^{-12} M (picomolar), and even as low as 10^{-13} M (sub-picomolar).

The assays of the present invention may be characterized as ECL assays; that is, in the assays of the present invention, the presence of analytes of interest, and preferably the quantity

of analytes of interest, is determined using ECL. Furthermore, the assays of the present invention rely on the use of ECL label in combination with certain classes of ECL quenchers. One class of such quenching moieties are those which comprise at least one benzene moiety. Sub-classes of such quenching moieties are those which comprise at least one phenol moiety, quinone moiety, benzene carboxylic acid, and/or benzene carboxylate moiety, as described above.

Thus, the present invention provides methods for detecting an analyte in a sample composition comprising the steps of:

(a) preparing an assay mixture comprising:

said sample composition;

a reagent having an ECL label; and

a reagent having an ECL quenching moiety, said ECL quenching moiety comprising at least one benzene moiety;

(b) determining any difference between the ECL emissions of

(i) the assay mixture prepared in step (a); and

(ii) an assay mixture comprising:

said reagent having an ECL label;

said reagent having an ECL quenching moiety; and

a known amount of said analyte; and

(c) correlating any difference determined in step (b) with the amount of analyte in said sample.

In one embodiment, said ECL quenching moiety comprises at least one moiety selected from the group consisting of phenol moieties, quinone moieties, benzene carboxylic acid moieties, and benzene carboxylate moieties, as described above. In another embodiment, said ECL quenching moiety comprises at least one phenol moiety. In another embodiment, said ECL quenching moiety comprises at least one quinone moiety. In another embodiment, said ECL quenching moiety comprises at least one benzene carboxylic acid moiety. In another embodiment, said ECL quenching moiety comprises at least one benzene carboxylate moiety.

In one embodiment, said known amount of analyte is zero.

In one embodiment, said reagent having an ECL label and said reagent having an ECL quenching moiety are the same reagent. In another embodiment, said reagent having an ECL label and said reagent having an ECL quenching moiety are different reagents.

In one embodiment, the method further comprises the initial step of conducting a chemical reaction on a substrate present in an initial sample composition to produce said analyte in said sample composition, and the final step of correlating any difference determined in step (b) with the amount of substrate in said initial sample composition.

In one embodiment, the method further comprises the step of conducting a chemical reaction with the assay mixture prepared in step (a) before the determining of step (b).

In one embodiment, the presence of a particular analyte of interest results in a *decrease* in ECL emission resulting from, for example, a decrease in a particular ECL emission from an ECL label. Such a change in ECL emission may result, for example, by introducing a quenching moiety into quenching contact with an ECL label. Alternatively, in another embodiment, the presence of a particular analyte of interest result in an *increase* in ECL emission resulting from, for example, an increase in a particular ECL emission from an ECL label. Such a change in ECL emission may result, for example, by removing a quenching moiety from quenching contact with an ECL label.

In one embodiment, the assays of the present invention exploit binding pairs in order to bring ECL labels and ECL quenching moieties together (into quenching contact) or apart (out of quenching contact). Examples of binding pairs include oligonucleotides and oligonucleotide hybridization probes; antibodies and antigens; enzymes and substrates; and strong binding pairs such as biotin-avidin. Such binding pairs may typically be employed in

assays of the present invention to permit the detection an analyte of interest which is one member of a binding pair, or which is conjugated to one member of a binding pair.

In one embodiment, the assays of the present invention may be employed to detect oligonucleotides (*e.g.*, DNA, RNA). Deoxyribonucleic acid (DNA) is a polynucleotide, more specifically, a polymer of deoxyribonucleotide units. A deoxyribonucleotide typically consists of a nitrogenous base, a sugar, and one or more phosphate groups. A deoxyribonucleoside typically consists of a nitrogenous base and a sugar. In naturally occurring DNA, the sugar group is typically β-D-2'-deoxyribofuranose and the nitrogenous base is typically a purine (*e.g.*, adenine, A, and guanine, G) or a pyrimidine (*e.g.*, thymine, T, or cytosine, C). Most commonly, the C-1 carbon of the D-2'-deoxyribose is attached to the N-1 of a pyrimidine or the N-9 of a purine; the configuration of this N-glycosidic linkage is β (the base lies above the plane of the sugar). The four naturally occurring deoxyribonucleosides are called deoxyadenosine (dA), deoxyguanosine (dG), deoxythymidine (dT), and deoxycytidine (dC). Deoxynucleotides are phosphate esters of deoxynucleosides. Most commonly, the phosphate ester is formed at the 5'-OH group of the sugar group (*i.e.*, the 5'-OH is converted to 5'-OPO_3^{-2}); the resulting compound is referred to as a nucleoside 5'-phosphate or a 5'-nucleotide. More than one phosphate group may be attached (*e.g.*, diphosphate, 5'-$OPO_2OPO_3^{-3}$; triphosphate, 5'-$OPO_2OPO_2PO_3^{-4}$). For example, an important activated precursor in the synthesis of DNA is deoxyadenosine 5'-triphosphate (dATP).

As mentioned above, DNA is a polymer of deoxyribonucleotide units. Most commonly, the polymeric backbone of DNA is constant and consists of deoxyribose groups linked by phosphate groups; more specifically, the 3'-position of one deoxyribose group (it was 3'-OH) is linked to the 5'-position of the adjacent deoxyribose group (it was 5'-OH) *via* a phosphodiester group (*i.e.*, -OP(=O)(O^-)O-). The variable aspect of DNA is its sequence of bases (*e.g.*, A, G, C, and T) attached at the 1'-position of each deoxyribose group. Thus, the four repeating units (often referred to as residues) most commonly found in DNA are referred to as deoxyadenylate, deoxyguanylate, deoxycytidylate, and deoxythymidylate.

A DNA polymer may be conveniently be represented by its component bases, often referred to as its "sequence." Since one end of the DNA molecule terminates in a sugar group having a free 3'-group (*e.g.*, 3'-OH, 3'-OPO_3^{-2}) and the other end terminates with a sugar group having a free a 5'-group (*e.g.*, 5'-OH, 5'-OPO_3^{-2}), it is necessary to unambiguously identify which end is which. As a matter of universal convention, DNA is recited left to right, from the 5'-terminus to the 3'-terminus. Thus, ACG denotes 5'-ACG-3' or 5'-A-3'-5'-C-3'-5'-G-3'. In some cases, a DNA polymer may be cyclic and thus have no terminus; in such cases, the sequence is recited from 5' to 3', from a suitable, possibly arbitrary, starting point.

DNA usually occurs in a double-helix form (Watson-Crick), wherein two helical polynucleotide chains (*e.g.*, strands) are coiled around a common axis, with each chain running in opposite directions ("anti-parallel") with respect to their 5'-3' polarity, as discussed above. The purine and pyrimidine bases are on the inside of the helix, whereas the phosphate and deoxyribose groups are on the outside. The planes of the bases are roughly perpendicular to the helix axis and the planes of the sugars are nearly parallel to the helix axis. The diameter of the helix is about 20 Å. Adjacent bases are separated by about 3.4 Å along the helix axis and are related by a rotation of about 36°. Thus, the helical structure repeats after ten residues on each chain, that is, at intervals of 34 Å. A relatively small DNA helix wherein each strand has 1000 residues is approximately 3.4 μm from end-to-end.

The two chains are held together by hydrogen bonding between pairs of bases (often referred to as "base pairs") and by stacking interaction (π-electron sharing) between adjacent base pairs. Because of steric and hydrogen bonding reasons, a purine is always paired with a pyrimidine; more specifically, adenine is always paired with thymine (*via* two hydrogen bonds), and guanine is always paired with cytosine (*via* three hydrogen bonds). Thus, each base pair contributes about 620 daltons to the molecular weight of the double helix. Note, however, that there is no restriction on the sequence of bases along the polynucleotide chain. It is the precise sequence of bases that carries genetic information.

The two strands of a DNA double helix readily come apart when the hydrogen bonds between its paired bases are disrupted, as may be accomplished by heating a solution of DNA or by adding acid or alkali to ionize the bases. The resulting unwinding of the double helix is commonly referred to as "melting" or "denaturation," and is characterized by a melting temperature at which half of the molecules are rendered single stranded. Melting is usually reversible, and the unwound chains may come together to reform the helix, in a process commonly referred to as "annealing," "renaturation," or "hybridization."

Ribonucleic acid (RNA) is another example of a polynucleotide. Like DNA, RNA is a polymer consisting of nucleotides jointed by a 3'-5' phosphodiester bonds. The covalent structure of RNA differs from that of DNA in two important respects. In RNA, the sugar group is β-D-ribose (instead of β-D-2'-deoxyribose). Also, one of the four major bases in RNA is the pyrimidine uracil, U (which replaces thymine found in DNA). Thus, in RNA, base pairs are AU and GC (instead of AT and GC found in DNA). RNA can be single-stranded or double-stranded, though usually it is single stranded. Although RNA cannot form a double helix of the B-DNA type, RNA often forms regions of double-helical structure produced by self-hybridization and the formation of hairpin loops.

DNA may be replicated with the aid of an enzyme, referred to as a DNA polymerase (*e.g.*, DNA pol α, β, γ, δ, ε). Typically, a DNA polymerase catalyzes the step-by-step addition of deoxyribonucleotides units to the 3'-terminus of a pre-existing DNA chain (often referred to as a primer) according to a template (typically a single strand of DNA) to which the primer has been hybridized. Typically, the chain-elongation reaction catalyzed by DNA polymerase is a nucleophilic attack of the 3'-OH terminus of the primer on the innermost (*i.e.*, α-phosphorus) phosphorus atom of a deoxyribonucleoside triphosphate; a phosphodiester bridge is formed and pyrophosphate concomitantly released. The DNA polymerase catalyzes the formation of the phosphodiester bond only if the base on the incoming nucleotide is complementary to the base on the template strand; indeed, mismatched base pairs are removed. In this way, the template driven replication proceeds with very high fidelity and with an error rate of less than 10^{-8} per base pair.

Genes comprise DNA. A particular DNA sequence encodes a particular amino acid sequence. In this way, proteins (poly amino acids, polypeptides) are encoded by DNA. DNA, preferably double stranded DNA, is used as a template for an RNA polymerase (*e.g.*, RNA pol I, II, III) to produce messenger RNA (mRNA) which encodes a particular protein. In this way, DNA is transcribed into mRNA. Triplets of mRNA residues, referred to as "codons," represent each of the 20 naturally occurring amino acids according to the genetic code. The mRNA is then itself used as a template and is "threaded" through a ribosome (comprised of ribosomal RNA, rRNA, and ribosomal proteins) to produce the protein encoded by the particular mRNA. In this way, mRNA is translated into protein. Individual amino acids, which are attached to a short piece of transfer RNA (tRNA) which also recognizes a specific codon in the mRNA, are incorporated into the growing protein by the ribosome. Coded DNA (cDNA) may be obtained from mRNA (acting as a template) using the enzyme reverse transcriptase. In this way, coding a particular protein may be obtained in a DNA form which is often more suitable for cloning and other genetic manipulations.

Polymerase chain reaction (PCR), developed in the mid-1980's, permits the simple and rapid production of large quantities of a specified DNA sequence without resorting to cloning. PCR exploits the ability of DNA polymerases (*e.g.*, *Taq* polymerase) to replicate DNA from a single stranded template DNA. Both DNA strands can serve as templates; single stranded templates may be easily produced, for example, by heating double-stranded DNA to a temperature near boiling. PCR requires that certain reagents be present in the reaction mixture, including activated nucleotide monomers (*e.g.*, ATP, GTP, CTP, TTP) and Mg^{+2}. PCR also requires a small piece of double stranded DNA at which to initiate (*i.e.*, prime) replication, which is usually provided by annealing (hybridizing) a suitable oligonucleotide "primer" at the site from which replication is to begin. Since DNA polymerase replicates DNA in the 3' to 5' direction, both strands may act as templates if two primers are provided, one which will hybridize to one strand, and one which will hybridize to the other strand. Following replication, the newly grown double stranded DNA (comprising the template strand and newly grown strand) is melted (*e.g.*, by heating to near boiling), and each of the resulting

single strands may act as a template in the next cycle. In this way, each cycle effectively doubles the number of desired single-stranded DNA fragments, and increases the proportion of DNA fragments which are identical (as defined by the positions of the two primers).

PCR is readily adapted to automation. Typically, a DNA sample is initially heated (*e.g.*, 94°C, 5 min) to separate the strands, and the reagents (*e.g.*, *Taq* polymerase, primers, excess activated nucleotide monomers, Mg^{+2}, *etc.*). In a first heating step (*e.g.*, 30-65°C, 30 s), primers bind to the DNA strands. In a second heating step (*e.g.*, 65-75°C, 2-5 min), the polymerase synthesizes new DNA strands. In a third heating step (*e.g.*, 94°C, 30 s), the strands of the resulting double stranded DNA are separated. The three steps are repeated for each cycle. Typically, from 10-60 cycles are performed. Theoretically, 32 cycles will yield approximately 10^9 copies of the desired double stranded DNA fragment.

Specific oligonucleotides (*e.g.*, DNA, RNA) may often be synthesized directly from monomers, dimers, *etc.* without the aid of a polymerase and without the need for a template strand. Typically, a solid-phase method is employed in which nucleotides are added to a nascent oligonucleotide which is attached to a solid support. A number of solid-phase oligonucleotide syntheses are known, including triester, phosphite, and phosphoramidate methods, though the last is often the preferred.

Typically, solid-phase oligonucleotide synthesis by the phosphoramidate method involves stepwise synthesis of the oligonucleotide in the 5'-direction by reiteratively performing four steps: deprotection, coupling, capping, and oxidation. In the first step ("deprotection"), the growing oligonucleotide, which is attached at the 3'-end *via* a 3'-O-group to a solid support, is 5'-deprotected to provide a reactive group (*i.e.*, a 5'-OH group). In the second step ("coupling"), the 5'-deprotected supported oligonucleotide is reacted with the desired nucleotide monomer, which itself has first been converted to a 5'-protected, 3'-phosphoramidite. For example, the 5'-OH group may be protected in the form of a 5'-ODMT group (where DMT is 4,4'-dimethoxytrityl) and the 3'-OH group may converted to a 3'-phosphoramidite, such as $-OP(OR')NR_2$, where R is the isopropyl group,

$-CH(CH_3)_2$, and R' is, for example, -H (yielding a phosphoramidite diester), or $-CH_3$, $-CH_2CH_3$, or the beta-cyanoethyl group, $-CH_2CH_2CN$ (yielding a phosphoramidite triester). The 3'-phosphoramidite group of the monomer reacts with the deprotected 5'-OH group of growing oligonucleotide to yield the phosphite linkage 5'-OP(OR')O-3'. Not all of the growing oligonucleotides will couple with the provided monomer; those which have not "grown" would yield incomplete oligonucleotides and therefore must be withdrawn from further synthesis. This is achieved by the third step ("capping"), in which all remaining -OH groups (*i.e.*, unreacted 5'-OH groups) are capped, for example, in the form of acetates ($5'-OC(O)CH_3$) by reaction with acetic anhydride. Finally, in the oxidation step, the newly formed phosphite group (*i.e.*, 5'-OP(OR')O-3') of the growing oligonucleotide is converted to a phosphate group (*i.e.*, 5'-OP(=O)(OR')O-3'), for example, by reaction with aqueous iodine and pyridine. The four-step process may then be reiterated, since the oligonucleotide obtained after oxidation remains 5'-protected and is ready for use in the first deprotection step described above. When the desired oligonucleotide has been obtained, it may be cleaved from the solid support, for example, by treatment with alkali and heat. This step may also serve to convert phosphate triesters (*i.e.*, when R' is not -H) to the phosphate diesters ($-OP(=O)_2O-$), as well as deprotect base-labile protected amino groups of the nucleotide bases.

Most methods for detecting specific DNA and RNA sequences rely on nucleic acid hybridization. Typically, such methods rely on the formation of a duplex between a target DNA or RNA sequence and a labeled nucleic acid hybridization probe. Hybridization probes are usually complementary to a specific part of the target nucleic acid. Note, however, that a hybridization probe may be only partially complementary, yet still form a stable duplex with the target sequence. Typically, hybridization probes are at least 70% complementary, though more often at least 90% complementary to the target sequence. Hybridization probes usually have a sequence which is long enough to ensure both selective and stable hybridization. Typically, hybridization probes have from 6 to about 500 monomer units (*e.g.*, nucleotides), though more typically from about 10 to about 100 monomer units. Specific hybridization probes having the desired sequence are often synthesized directly using solid-phase oligonucleotide synthesis methods. Labeled nucleic acid probes are utilized in a variety of

assay formats including dot blots, Southern blots (DNA target), Northern blots (RNA target), in situ hybridization, plaque hybridization, and colony hybridization. A number of different substances have been used to label a nucleic acid probe, and a number of different methods have been used to detect these labels. See, for example, Kricka, 1992.

In one embodiment, the assays of the present invention may be employed to detect DNA by employing two oligonucleotide hybridization probes, one with an attached ECL label and the other with an attached ECL quenching moiety. The probes may be specifically chosen so that, upon annealing with the target DNA, the ECL label and ECL quenching moiety are brought into quenching contact, thereby reducing the observed ECL emission. Thus, a decrease in ECL emission may be correlated with the amount of target DNA present in the sample.

In another embodiment, the assays of the present invention may be employed to detect DNA by employing a oligonucleotide hybridization probe which possesses both an ECL label and an ECL quenching moiety, in combination with a DNA polymerase (typically in a non-separation assay). For example, a target DNA in a sample may be detected by forming a mixture comprising the sample, a suitable oligonucleotide hybridization probe having both an ECL label (L) and an ECL quenching moiety (Q), a suitable oligonucleotide primer which hybridizes to the target DNA at a position upstream from the hybridization probe, a 5'-specific DNA *exo*-polymerase, such as the well known *Taq* polymerase (derived from *Thermus Aquaticus*), and suitable concentrations of activated nucleotide monomers (*e.g.*, ATP, GTP, CTP, TTP) and other reagents (*e.g.*, KCl, Tris HCl, $MgCl_2$). Typically, the ECL label and ECL quenching moiety of the unhybridized oligonucleotide hybridization probe are in quenching contact, and low ECL emission is observed. The mixture is processed to permit the hybridization probe and primer to anneal to the target DNA. Typically, the ECL label and ECL quenching moiety of the hybridized probe are in quenching contact, and, again, low ECL emission is observed. The polymerase reaction is then allowed to proceed. Starting at the 3'-end of the primer, the 5'-specific DNA *exo*-polymerase extends the primer in the 5'-direction, one nucleotide at a time, using the target DNA as a template. If, as the

polymerase proceeds downstream in the 5'-direction, it encounters a bound hybridization probe, the polymerase will degrade the bound probe as it extends the primer. The hybridization probe is converted to short oligonucleotide fragments (typically monomers), which are freed from the target DNA and enter the solution mixture. Since the ECL label and the ECL quenching moiety were attached to different monomer units, they are, upon degradation, freed into solution and thus are no longer held in quenching contact. Thus, an increase in ECL emission may be correlated with the amount of target DNA present in the sample. An analogous method has been illustrated for fluorescence labels and fluorescence quenchers. See, for example, Wittwer, 1997.

In yet another embodiment, the assays of the present invention may be employed to detect DNA by employing a oligonucleotide hybridization probe which possesses both an ECL label and an ECL quenching moiety, and which has self-hybridization sequences. In the absence of the target DNA, the probe self-hybridizes (typically forming a hairpin or hairpin-loop structure), bringing the ECL label and the ECL quenching moiety into quenching contact. In the presence of the target DNA, the probe preferentially anneals to the target DNA, and in doing so, separates the ECL label from the ECL quenching moiety so that they are no longer in quenching contact, and the ECL emission increases. Thus, an increase in ECL emission may be correlated with the amount of target DNA present in the sample.

In one embodiment, the assays of the present invention may be employed as immunoassay to detect antibodies or antigens. Antibodies, also referred to as immunoglobulins, are proteins synthesized by an animal in response to the presence of a foreign substance. They are secreted by plasma cells, which are derived from B lymphocytes (B cells). These soluble proteins are the recognition elements of the humoral immune response. Each antibody has a specific affinity for the foreign material that stimulated its synthesis, and readily binds with the foreign material to form a complex. A foreign macromolecule capable of eliciting antibody formation is called an antigen (or immunogen). Proteins, polysaccharides, and nucleic acids are usually effective antigens. The specific affinity of an antibody is not for the entire macromolecular antigen, but instead for a particular

site on the antigen called the antigenic determinant (or epitope). Most small molecules do not stimulate antibody formation. However, they can elicit the formation of specific antibodies if they are attached to macromolecules. The macromolecule is then the carrier of the attached chemical group, which is called a haptenic determinant. The small foreign molecule by itself is called a hapten. Antibodies elicited by attached haptens will bind unattached haptens as well.

Structurally, antibodies consist of four individual protein chains: two light (L) chains of molecular weight about 17,000 daltons, and two heavy (H) chains of molecular weight about 35,000, which are held together by disulfide bonds. In humans, there are five classes of heavy chains, μ, δ, γ, ε, and α, and two classes of light chains, κ and λ. It is the class of heavy chain which characterizes the antibody (*i.e.*, immunoglobulin, Ig) as an IgM, IgD, IgG, IgE, or IgA, respectively. Generally, each of the light and heavy chains consist of a variable region (at the amino end) and a constant region (at the carboxy end), though the heavy chain's constant region is often sub-divided into domains. Usually, it is the amino acid sequence of the variable regions of the light and heavy chains which determine the specificity of antigen binding; thus each antibody usually has two antigen binding sites. Generally, antibodies may also be structurally described according to their products upon enzymatic degradation, for example, by papain, pepsin, or trypsin. Typically, upon digestion by the enzyme papain, two Fab fragments (each having one complete light chain and part of one heavy chain; each has one antigen binding site) and one Fc fragment (having the remainder of each of the two heavy chains, and having no antigen binding sites) are obtained. Typically, upon digestion by the enzyme pepsin, one $F(ab')_2$ fragment (having two complete light chains and one part of each heavy chain; has two antigen binding sites) and one pFc' fragment (having the remainder of each of the two heavy chains, and having no antigen binding sites) are obtained.

Each antibody-producing cell produces only one type of antibody, and the specific type of antibody produced by a given cell is related to that cell's initial interaction with the antigen. In this way, when a foreign substance is introduced into an animal, a large number of different antibodies are produced, with varying binding specificity for the antigen. B lymphocytes, the

precursors of plasma cells, are triggered to divide and proliferate by the binding of antigen to receptors, antibody molecules which span the membrane and are have binding sites exposed on the cell surface. The soluble antibodies subsequently produced by the activated cell have the same specificity as the membrane-bound antibody.

Antibodies may be generated, for example, by administering an antigen to an animal. Typically, the antigen comprises a hapten bound to a hapten determinant (*e.g.*, a carrier macromolecule), such as serum albumin, serum globulins, lipoproteins, and the like. The antigen may be conveniently prepared for injection by rehydrating lyophilized antigen to form a solution or suspension, and is usually mixed with an adjuvant. Examples of adjuvants include water-in-oil emulsions, such as Freund's complete adjuvant for the first administration, and Freund's incomplete adjuvant for booster doses. Typically, the antigen composition is administered at a variety of sites, and in two or more doses over a course of at least about 4 weeks.

Serum (*i.e.*, polyclonal antiserum) is harvested from the animal and tested for the presence of desired antibody using the antigen or an antigen analog in a standard immunoassay or precipitation reaction. The polyclonal antiserum will typically contain some antibodies which are not reactive with the antigen, and some which are reactive with the antigen but are also cross-reactive with other antigens (*e.g.*, not highly selective). Methods for purifying specific antibodies from a polyclonal antiserum are known in the art. A particularly effective method is known as affinity purification which employs a column having antigen conjugated to a solid phase (*e.g.*, a Sepharose column). The polyclonal antisera is passed over the column, the column washed, and the desired antibody eluted with a mild denaturing buffer. For general techniques used in raising, purifying and modifying antibodies, and the design and execution of immunoassays, see, for example, Weir *et al.*, 1996; Coligan *et al.*, 1991; Wild, 1994; and Masseyeff *et al.*, 1993.

Since a given antibody-producing cell (*e.g.*, a splenocyte) produces only one specific antibody, it is usually necessary to clone that cell in order to generate quantities of that specific

antibody, for example, by fusing the antibody-producing cell with a non-antibody producing myeloma cell (a cell produced by multiple myeloma, a malignant disorder of antibody-producing cells). Fusion may be achieved, for example, by exposing the cells to polyethylene glycol, but more usually achieved by transfection with Epstein Barr Virus, or transformation with oncogenic DNA.

Unlike the antibody producing cell, the fused cell retains the neoplastic character of the myeloma cell, and thus proliferates in culture; in this way, the antibody-producing cell is immortalized. Typically, many antibody-producing cells are cloned and cultured, and those clones that produce antibodies of the of the desired specificity are selected. Specificity is typically determined from culture supernatants, for example, by the antigen as the detecting reagent in an immunoassay. A supply of the desired monoclonal antibody from the selected clone can then be purified from a large volume of culture supernatant, or from the ascites fluid of suitably prepared host animals injected with the clone. The antibody may optionally be purified using standard biochemical preparation techniques such as ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. In another method, antibody-producing cells may be harvested from an immunized animal donor, or they can be harvested from an unimmunized donor and pre-stimulated in vitro by culturing in the presence of antigen and immunostimulatory growth factors. Cells which produce antibody of the desired specificity can be selected by contacting with antigen under conditions which result in proliferation of specific clones but not non-specific clones. For general techniques pertaining to monoclonal antibodies and hybridomas, see, for example, Harrow & Lane, 1988; Wands *et al.*, 1985; Milstein *et al.*, 1984; and Hoffmann, 1984.

Thus, the term "antibody," as used herein, relates to both polyclonal and monoclonal antibody, and encompasses not only intact antibody molecules, but also such antibody fragments and antibody derivatives (as may be prepared by techniques known in the art) which retain the antibody activity of an intact immunoglobulin. In this context, "antibody activity" relates to the ability of an antibody to bind a specific antigen in preference to other potential antigens *via* the antibody's antigen binding site. Fragments and other derivatives of antibodies

can be prepared by methods of standard protein chemistry, such as subjecting the antibody to cleavage with a proteolytic enzyme like pepsin, papain, or trypsin; and reducing disulfide bonds with such reagents as dithiothreitol. Genetically engineered variants of intact antibodies can be produced by obtaining a polynucleotide encoding the antibody, and applying the general methods of molecular biology to splice encoding sequences or introduce mutations and translate the variant. Antibodies that are engineered variants of particular interest include chimeric and humanized antibodies, Fab-like fragments, single-chain variable region fragments (scFv), and diabodies.

Although antibodies are usually screened or purified according to their ability to react with the antigen, they are often also screened according to other criteria, such as low cross-reactivity with potential interfering substances; antibody-antigen reaction rates and antibody-antigen affinity, both of which may affect the sensitivity and capacity of the antibody-antigen system; and the titer of antibody produced by a biological source. Ultimate selection of an antibody may require a compromise between these various features.

In one embodiment, the assays of the present invention may be employed as immunoassay to detect an antibody or an antigen. For example, a target antibody (to be detected) in a sample may first be derivatized to possess one or more ECL quenching moieties (using, for example, quenching agents with amino-reactive groups). An antigen or antigen analog may then be prepared which possesses an ECL label. Upon mixing, antibody-antigen complexes are formed wherein the ECL label and the ECL quenching moiety are brought into quenching contact, thereby reducing the observed ECL emission. Thus, a decrease in ECL emission may be correlated with the amount of target antibody present in the sample. Alternatively, target antibodies may be derivatized to possess ECL labels, and the antigen or antigen analog may be prepared to possess ECL quenching moieties. Analogous methods may be used to detect target antigens.

In another embodiment, the assays of the present invention may be employed as a competition immunoassay to detect an antibody or an antigen. For example, a target antibody

(to be detected) in a sample may first be derivatized to possess one or more ECL quenching moieties (using, for example, quenching agents with amino-reactive groups). An antigen or antigen analog may then be prepared which possesses an ECL label. Upon mixing, antibody-antigen complexes are formed wherein the ECL label and the ECL quenching moiety are brought into quenching contact, thereby reducing the observed ECL emission. A second antigen or antigen analog, which lacks an ECL label, and which has a similar binding affinity for the target antibody may be added. (Alternatively, the first antigen may be unlabeled and the second antigen may be labeled.) In this way, the unlabeled antigen competes with the labeled antigen; labeled antigen which is freed upon competition will increase the ECL emission. Thus, a change in ECL emission may be correlated with the amount of target antibody present in the sample. Analogous methods may be used to detect target antigens.

In one embodiment, the assays of the present invention may be employed to detect enzymes, enzyme agonists, and enzyme antagonists. Enzymes, the great majority of which are proteins (poly amino acids), are catalysts of biological systems. Enzymes typically offer substantial catalytic power (often accelerating reactions by a factor of 10^6 or more) and exquisite selectivity. By utilizing a full repertoire of intramolecular and intermolecular forces, enzymes are able both to bring substrates into optimal orientation for making and breaking chemical bonds, and to stabilize the transition states for the desired reaction path. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions, with a very low proportion of side-reactions which yield undesired by-products, and with a very high degree of selectivity (often virtually absolute).

The first step in enzymatic catalysis involves the formation of an enzyme-substrate complex, wherein the substrate is typically bound to a specific region of the enzyme usually referred to as the active site. The active site typically occupies a relatively small portion of the total volume of an enzyme, and many of the amino acid residues in an enzyme are not in contact with the substrate. The active site is a three-dimensional entity, typically formed by chemicals groups on different amino acid residues (often far apart in a linear amino acid sequence) that come together as a result of the enzymes primary, secondary, tertiary, and

quaternary structure. Typically, substrates are bound to enzymes by multiple weak attractions (*e.g.*, electrostatic bonds, hydrogen bonds, van der Waals forces, hydrophobic interactions). In most cases, the active site is a crevice or cleft in the enzyme, into which a complementary substrate is bound. The specificity of binding typically depends on the arrangement of atoms in the active site. For example, the enzyme and substrate may be represented metaphorically by a lock and key, respectively, which have complementary structures. Alternatively, the enzyme and substrate may have complementary structures only after formation of an enzyme-substrate complex.

The activity of enzymes may often be increased or decreased by certain small molecules and ions (*e.g.*, drugs, toxins). Enzyme inhibitors typically reduce enzyme activity. An inhibitor may bind irreversibly at the active site, in which case the enzyme is rendered essentially permanently inactive. Alternatively, an inhibitor may bind reversibly at the active site, in which case the inhibitor, referred to as a competitive inhibitor, prevents the substrate from binding and competes with substrate for the binding site. Also, an inhibitor (typically referred to as a noncompetitive inhibitor or antagonist) may bind at a site other than the active site, and thereby reduce the enzyme's ability to bind substrate at the binding site. In contrast, molecules which increase enzyme activity (often referred to as agonists) typically bind at a site other than the active site, and thereby increase the enzyme's activity.

In one embodiment, the assays of the present invention may be employed to detect (or identify) an enzyme, substrate, irreversible inhibitor, competitive inhibitor, antagonist, or agonist. For example, an enzyme (to be detected) in a sample may first be derivatized to possess one or more ECL quenching moieties (using, for example, quenching agents with amino-reactive groups). An enzyme substrate may then be prepared which possesses an ECL label. Upon mixing, enzyme-substrate complexes are formed wherein the ECL label and one or more ECL quenching moieties are brought into quenching contact, thereby reducing the observed ECL emission. Thus, a decrease in ECL emission may be correlated with the amount of target enzyme present in the sample. Alternatively, target substrates may be derivatized to possess ECL labels, and the enzyme may be prepared to possess ECL quenching

moieties. Analogous methods may be used to detect target substrates, substrate analogs, irreversible inhibitors, competitive inhibitors, antagonists, and agonists.

In another embodiment, the assays of the present invention may be employed as a competition assay to detect, for example, an enzyme, substrate, substrate analog, competitive inhibitor, antagonist, or agonist. For example, to detect a substrate in a sample, one may first derivatize a suitable enzyme to possess one or more ECL quenching moieties (using, for example, quenching agents with amino-reactive groups). A substrate analog, which has a similar binding affinity for the enzyme as the substrate, and which possesses an ECL label, may then be prepared. Upon mixing the enzyme and substrate analog, enzyme-substrate analog complexes are formed wherein the ECL label and one or more ECL quenching moieties are brought into quenching contact. The sample, containing the substrate to be detected, is then added. The substrate, which lacks an ECL label, competes with the labeled substrate analog; labeled substrate analog which is freed upon competition will increase the ECL emission. Thus, an increase in ECL emission may be correlated with the amount of target substrate present in the sample. Analogous methods may be used to detect enzymes, substrate analogs, competitive inhibitors, antagonists, and agonists.

In one embodiment, the assays of the present invention may be employed to detect materials which may be selectively derivatized to possess one member of a strong binding pair. Examples of strong binding pairs include biotin-avidin and biotin-avidin analogs, such as biotin-streptavidin. Biotin, also known as vitamin H of the vitamin B complex, is an imidazole pentanoic acid of empirical formula $C_{10}H_{15}O_3N_2S$. Avidin, a 70 kilodalton protein found in egg white, has a very high binding affinity for biotin. Streptavidin, a similar protein found in the bacteria, *Streptomyces avidinii*, has an even higher binding affinity for biotin, partially due to its four biotin binding sites.

For example, a target molecule (to be detected) may be selectively derivatized to possess both a biotin moiety (using, for example, a commercially available biotinylating agent) and an ECL label. A streptavidin derivative may be prepared which possesses one or more

quenching moieties. Upon mixing, biotin-streptavidin complexes are formed wherein the ECL label and one or more ECL quenching moieties are brought into quenching contact, thereby reducing the observed ECL emission. Thus, a decrease in ECL emission may be correlated with the amount of target molecule present in the sample. Alternatively, the target molecule may be selectively derivatized to possess, for example, biotin/ECL quenching moiety, avidin/ECL label, or avidin/ECL quenching moiety; counterparts reagents would then comprise avidin/ECL label, biotin/ECL quenching moiety, and biotin/ECL label, respectively.

The present invention also provides reagents, reagent sets comprising one or more reagents, and reagent kits comprising one or more reagent sets, for use in the assay methods of the present invention. Reagents may be in solid, liquid, or gaseous form, though typically are in solid or liquid form. Examples of reagents include, but are not limited to, reagents for ECL labeling, reagents for attaching ECL quenching moieties, electrolyte compositions, solvents, and buffers. Reagents and/or sets of reagents for use in the assays of the present invention are typically provided in one or more suitable containers or devices. Reagent sets are typically presented in a commercially packaged form, as a composition or admixture where the computability of reagents will allow, as a reagent kit; for example, as a packaged combination of one or more containers, devices, or the like holding one or more reagents, and usually including written instructions for the performance of the assays.

D. Methods for Measuring ECL

A range of suitable apparati for measuring the ECL of sample are known in the art. See, for example, Blackburn *et al.*, 1991; Leland *et al.*, 1990; Hall *et al.*, 1991. Typically, ECL is measured using an apparatus which comprises (i) a receptacle for the sample (which is typically a liquid); (ii) two or more electrodes disposed in the receptacle and in contact with the composition to be examined, one of which is the "working electrode" at which electrochemiluminescent species are produced, and (iii) a detector, which detects some fraction of the photons emitted during electrochemiluminescence.

For convenience, the ECL apparatus typically has three electrodes: a working electrode, a counter electrode, and a reference electrode. Often the reference electrode (*e.g.*, a standard Ag/AgCl electrode) is located some distance from, but in contact (*via* the electrolyte) with, the working and counter electrodes. The working and counter electrodes are typically noble or relatively inert metals such as platinum and gold.

The detector may be any device which detects (and preferably quantifies) photons, such as a photomultiplier tube (PMT), a photodiode, a charge coupled device, photographic or light sensitive film or emulsion. Typically, the detector is a PMT, which may be chosen to be particularly sensitive for a certain range of photons, for example, ultraviolet, visible, or infrared. The detector is typically positioned in a manner so that it may readily and efficiently detect the photons emitted during ECL. For example, in one embodiment, the working electrode is a gold or platinum disk, the PMT detector is positioned directly across from the front flat surface of the working electrode, and the composition to be examined flows laterally over the disk, between the disk and the PMT detector.

For convenience, the ECL apparatus typically incorporates means for fluid handling, including, for example, inlets to and outlets from the sample receptacle which are connected to reservoirs (*e.g.*, *via* tubing) for reagents, electrolyte/buffers, and the sample composition, and pumps (*e.g.*, a peristaltic pump) for moving liquids between the receptacle and the reservoirs. In this way, the apparatus may be used to measure ECL in either a static or flow-through configuration.

A well known and commercially available ECL apparatus is the Origen I Analyzer®, which integrates a photometer (as detector), an electrochemical cell (receptacle and electrodes), a potentiostat (for operating the electrochemical cell), and means for fluid and sample handling. The analyzer employs a flow injection system that permits rapid and reproducible determinations of sequential samples. The photometer is a photomultiplier tube (typically red-sensitive for optimal detection of $Ru(bpy)_3^{2+}$ labels) positioned directly above

the working electrode so that the light from the electrode is recorded and integrated during each measurement.

As discussed above, ECL is the emission of photons of electromagnetic radiation (*e.g.*, light) from an electronically excited chemical species which has been generated electrochemically. Thus, to measure the ECL of a particular sample, the sample must be electrolyzed to produce electro-oxidized and/or electro-reduced species which, either directly and following further reaction, emit photons. The sample is typically electrolyzed by applying an electrical potential to the working electrode, for example, with a battery or other source of electromotive force (EMF). For convenience, the potential difference is reported as the potential of the working with respect to the reference electrode, with electrochemical current (faradaic current) flowing between the working and auxilliary electrodes. Thus, the working electrode potential typical ranges from -10.00 to +10.00 V, though more commonly from -6.00 to +6.00 V, and even more commonly from -3.00 to +3.00 V. The working electrode potential may be static, may alternate, or may reflect a more complex function. Means for applying a particular electric potential (*e.g.*, waveform) are well known in the electrochemical arts. See, for example, Kamin *et al.*, 1992. The potential which must be applied to the working electrode in order to produce ECL is a function of the exact chemical species which are involved in the ECL reaction sequence as well as other factors such as the pH of the sample composition and nature of the electrode. It is well known to those of skill in the art of ECL how to determine both the optimal potential to produce ECL as well as the optimum wavelength at which to detect the ECL.

Again, in order to measure the ECL of a particular sample, the sample must be electrolyzed to produce electro-oxidized and/or electro-reduced species which, either directly or following further reaction, emit photons. To effect optimum electrolysis, ions should be present in the sample composition which may migrate between the working electrode and the counter electrode, thereby effecting the transfer of charge. Therefore, in order to facilitate the ECL measurement, the sample is typically mixed with an ECL assay media (*e.g.*, ECL assay

buffer) which comprises ions which will effect the transfer of charge during the ECL measurement but will not interfere with the ECL reaction sequence.

The term "ECL assay media," as used herein, pertains to a composition which is optionally (though usually) mixed with the sample prior to performing the ECL measurement. Generally, the ECL assay media is a fluid, though more typically is a liquid, and comprises one or more dissolved salts. Typically, the ECL assay is a liquid and comprises one or more solvents and one or more dissolved salts. Typically, the salts are present in millimolar concentrations.

In one embodiment, the ECL assay comprises water (*i.e.*, H_2O) and one or more dissolved salts. Examples of water soluble salts include chloride salts such as NaCl, KCl, $N(C_4H_9)_4Cl$; bromide salts such as NaBr, KBr, $N(C_4H_9)_4Br$; nitrate salts such as KNO_3, $NaNO_3$, and $N(C_4H_9)_4NO_3$; phosphate salts such as Na_3PO_4, K_3PO_4, Na_2HPO_4, K_2HPO_4, NaH_2PO_4, and KH_2PO_4; and sulfate salts such as Na_2SO_4, and K_2SO_4.

In another embodiment, the ECL assay comprises one or more organic solvents and one or more dissolved salts. Examples of suitable organic solvents include acetonitrile (*i.e.*, CH_3CN, ACN), dimethylsulfoxide (*i.e.*, $(CH_3)_2SO$, DMSO), N,N-dimethylformamide (*i.e.*, $(CH_3)_2NCHO$, DMF), methanol (*i.e.*, CH_3OH), and ethanol (*i.e.*, C_2H_5OH). Examples of salts which are soluble in typical organic solvents include tetrabutylammonium salts, such as tetrabutylammonium tetrafluoroborate (*i.e.*, $(C_4H_9)_4NBF_4$).

In some embodiments, and particularly in those wherein the ECL assay media comprises water, the ECL assay media is pH buffered. For example, an aqueous ECL assay media may conveniently be pH buffered by the addition of a phosphate (*e.g.*, KH_2PO_4, typically at about 0.01 to 0.05 M) followed by adjusting the pH to a desired value (*e.g.*, physiological pH 7.2) by the addition an appropriate amount of a suitable strong acid (*e.g.*, HCl) or strong base (*e.g.*, NaOH). Once buffered, the pH of the ECL assay media is

relatively insensitive to small changes in its chemical composition, such as those which may occur during the ECL measurement.

The ECL assay media may also comprise one or more ECL coreactants, which take part in the chemical reactions involving the electro-oxidized and/or electro-reduced species, the final result being the emission of a photon (*i.e.*, ECL). The term "ECL coreactant," or more simply "coreactant," as used herein, pertains to a chemical compound which, either itself or its electrochemical reduction/oxidation product(s), plays a role in the ECL reaction sequence.

Often coreactants permit the use of simpler means for generating ECL (*e.g.*, the use of only half of the double-step oxidation-reduction cycle) and/or improved ECL intensity. In one embodiment, coreactants are chemical compounds which, upon electrochemical oxidation/reduction, yield, either directly or upon further reaction, strong oxidizing or reducing species in solution. An example of a coreactant is peroxodisulfate (*i.e.*, $S_2O_8^{2-}$, persulfate) which is irreversibly electro-reduced to form oxidizing $SO_4\bullet^-$ ions. Another example of a coreactant is oxalate (*i.e.*, $C_2O_4^{2-}$) which is irreversibly electro-oxidized to form reducing $CO_2\bullet^-$ ions. An example of a class of coreactants which act as reducing agents are amines or compounds containing amine groups, including, for example, tri-*n*-propylamine (*i.e.*, $N(CH_2CH_2CH_3)_3$, TPAH).

Examples of coreactants include, but are not limited to, lincomycin; clindamycin-2-phosphate; sparteine; erythromycin; 1-methylpyrrolidone; N-ethylmorpholine; diphenidol; atropine; trazodone; 1-ethylpiperidine; hydroflumethiadize; hydrochlorothiazide; clindamycin; tetracyline; streptomycin; gentamycin; reserpine; trimethylamine; tri-*n*-butylamine; triethanolamine; piperidine; 1,4-piperazine bis(ethanesulfonic acid); tri-*n*-butylphosphine; N,N-dimethylaniline; pheniramine; bromopheniramine; chloropheniramine; diphenylhydramine; di-*n*-propylamine; 2-dimethylaminopyridine; pyrilamine; 2-benzylaminopyridine; leucine; valine; glutamic acid; phenylalanine; alanine; arginine; histidine; cysteine; tryptophan; tyrosine; hydroxyproline; asparagine; methionine; theonine;

serine; cyclothiazine; trichloromethiazide; 1,3-diaminopropane; piperazine, chlorothiazide; hydrozinothalanzine; barbituric acid; persulfate; nicotinimide adenine dinucleotide; penicillin; 1-piperidinyl ethanol; 1,4-diazbicyclo(2.2.2)octane; 1,4-diaminobutane; 1,5-diaminopentane; 1,6-diaminohexane; ethylenediamine; ethylenediamine tetraacetic acid; benzenesulfonamide; tetramethylsulfone; ethylamine; *n*-propylamine; *n*-butylamine; *s*-butylamine; *t*-butylamine; *n*-pentylamine; *n*-hexylamine; oxalic acid; hydrazine sulfate; glucose; methylacetamide; and phosphoroacetic acid.

The concentration of coreactant in the sample composition varies according to the specific coreactant chosen, and one of ordinary skill in the art is readily able to determine a suitable concentration. Typically, the coreactant concentration is chosen to be approximately 1000 times greater than the concentration of ECL label.

The ECL assay media may also comprise one or more ECL enhancers, which may increase ECL emission and may also serve as surfactants or wetting agents to prevent or reduce adsorption on the electrode and/or interior walls of the ECL apparatus. A number of ECL enhancers are well known in the art. See, for example, Shah *et al.*, 1990. One group of ECL enhancers may be described as *para*-substituted benzenes wherein one substituent (R_1) is hydrogen or a $C_{1\text{-}20}$ alkyl group and the other (*para*) substituent (R_2) is a poly(alkoxy) alcohol of the formula $-[O-(CH_2)_n]_mOH$ where n is an integer from 1 to 20 and m is an integer from 0 to 70. One ECL enhancer, which is commercially available under the name Triton X-100®, has R_1 as $-C(CH_3)_2CH_2C(CH_3)_3$ and R_2 as $-(O-CH_2CH_2)_{9\text{-}10}OH$. Another ECL enhancer, which is commercially available under the name Triton X-401®, has R_1 as $-C_9H_{19}$ and R_2 as $-(O-CH_2CH_2)_{40}OH$. When utilized, the ECL enhancer is generally present in an amount which increases the ECL emission. Typically, the amount is from about 0.01 to about 5% (v/v), and often from about 0.1 to about 1% (v/v).

E. Examples

Several embodiments of the present invention are described in the following examples, which are offered by way of illustration and not by way of limitation.

Example 1

$Ru(bpy)_3^{+2}$/TPAH ECL Quenching by Phenol.

An appropriate amount of tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate (*i.e.*, $Ru(bpy)_3Cl_2.6H_2O$, Aldricn® Chemical Co.) was dissolved in Elecsys® buffer solution (flash-ECL assay buffer number 1518-001; a phosphate based buffer with 0.18 M tri-*n*-propylamine (TPAH) and Thesit® as a wetting agent and ECL enhancer) and the solution diluted to yield a stock solution of 0.4 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.18 M TPAH (as coreactant) at pH = 6.8. Microliter amounts of 1 M phenol (*i.e.*, C_6H_5OH, ultrapure, Clontech®) (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 15 mM.

ECL intensity was measured and recorded (in arbitrary units, *e.g.*, counts) for each of the samples using a commercially available electrochemiluminescence analyzer, the Origen I Analyzer®, which integrates a photometer, a potentiostat, an electrochemical cell, and means for fluid and sample handling. The analyzer employs a flow injection system that permits rapid and reproducible determinations of sequential samples. The photometer is a red-sensitive photomultiplier tube positioned directly above the working electrode so that the light generated at or near the electrode is recorded and integrated during each measurement. Typically, an oxidative electrochemical sequence/potential was applied to the working electrode, and light intensity measured with a photomultiplier tube using standard Origen® parameters. Typically, the potential was ramped from 0 to 2800 mV at a sweep rate of 4800 mV/s (frequency = 0.58 sec^{-1}). The electrode was cleaned prior to and after each run using a 0.176 M KOH buffered cleaning solution (Flash-ECL CS, from Boehringer Mannheim®,

identification number 1518470). An EG&G PAR Model 263A® Potentiostat/Galvanostat was used for all electrochemical measurements.

The data are illustrated in Figure 1. Note that the ECL signal for the sample having no phenol was greater than the detection capacity of the instrument, 10 million arbitrary units. Only 2 mM phenol resulted in the ECL being reduced to less than 7% of the ECL of the control sample. Only 5 mM phenol resulted in the ECL being reduced to less than about 0.01% of the ECL of the control sample.

This example demonstrates that micromolar concentrations of phenol effectively quench solution ECL of micromolar concentrations $Ru(bpy)_3^{2+}$ in the $Ru(bpy)_3^{2+}$/TPAH ECL reaction sequence. That is, upon electrochemical oxidation, the resulting excited state species, $Ru(bpy)_3^{2+*}$, is effectively quenched such that substantially less ECL intensity is observed as compared to the case where the quencher species is absent.

Example 2

$Ru(bpy)_3^{+2}/C_2O_8^{-2}$ ECL Quenching by Phenol.

Appropriate amounts of $Ru(bpy)_3Cl_2.6H_2O$ and $Na_2C_2O_8$ (Aldrich Chemical Company) were dissolved in phosphate buffered saline (*i.e.*, "PBS"; 50 mM Na_3PO_4, 100 mM NaCl, pH 7.0, 0.2 μm filtered) and diluted to yield a stock solution of 0.4 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 200 mM $C_2O_8^{-2}$ (as coreactant) at pH = 7.0. Microliter amounts of 1 M phenol (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 20 mM. ECL intensity was measured and recorded (in arbitrary units) for each of the samples. The data are illustrated in Figure 2. 2 mM phenol resulted in the ECL being reduced to less than 6% of the ECL of the control sample. 8 mM phenol resulted in the ECL being reduced to less than about 0.1% of the ECL of the control sample.

This example demonstrates that micromolar concentrations of phenol effectively quench solution ECL of sub-micromolar concentrations $Ru(bpy)_3^{2+}$ in the $Ru(bpy)_3^{2+}/C_2O_8^{-2}$ ECL reaction sequence. Although the ECL intensity of the $Ru(bpy)_3^{2+}/C_2O_8^{-2}$ system is intrinsically lower than that of the $Ru(bpy)_3^{2+}$/TPAH system (by a factor of about 10-50), the use of phenol in the $C_2O_8^{-2}$ system did yield approximately 5% higher quenching efficiency.

Example 3

$Ru(bpy)_3^{+2}$ ECL Quenching by *p*-Hydroxybenzoic Acid and *p*-Aminobenzoic Acid.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ was dissolved in Elecsys® buffer solution and diluted to yield a stock solution of 0.3 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.18 M TPAH (as coreactant) at pH = 6.8. Microliter amounts of 1 M *p*-hydroxybenzoic acid (*i.e.*, HOC_6H_4COOH, PHBA, 99+% purity, Aldrich Chemical Company) or 1 M *p*-aminobenzoic acid (*i.e.*, $H_2NC_6H_4COOH$, PABA, 99+% purity, Aldrich Chemical Company) (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with quenching agent concentrations ranging from 2 to 10 mM. For comparison, microliter amounts of 1 M phenol (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 10 mM. ECL intensity was measured and recorded (in arbitrary units) for each of the samples. The data are illustrated in Figure 3.

This example demonstrates that, at comparable concentrations, phenol quenches the ECL of micromolar concentrations $Ru(bpy)_3^{2+}$ in the $Ru(bpy)_3^{2+}$/TPAH ECL reaction sequence much more efficiently than either PHBA (by a factor of at least about 8) or PABA (by a factor of at least about 2). This example also demonstrates, via the known free radical scavengers PHBA and PABA, that interception of the TPA$^{\bullet}$ intermediate prior to formation of the $Ru(bpy)_3^{2+*}$ excited state is less likely than direct quenching of the excited state.

Example 4

$Ru(bpy)_3^{+2}$ ECL Quenching by Phenol Derivatives.

A number phenol derivatives, possessing one or more electron withdrawing and/or electron donating groups, were tested for their quenching efficiency in a manner analogous to that used in Example 3. Quenching agents (all >98% purity, from Aldrich Chemical Company) were dissolved to the appropriate concentration in ethanol, and appropriate aliquots were transferred to 1 mL aliquots of a $Ru(bpy)_3^{2+}$/TPAH stock solution. Those phenol derivatives which were tested as quenching agents included: *o*-cresol (*i.e.*, 2-methyl-phenol), *m*-cresol (*i.e.*, 3-methyl-phenol), *p*-cresol (*i.e.*, 4-methyl-phenol), *p*-fluorophenol, *m*-fluorophenol, *o*-fluorophenol, *o*-propylphenol, *p*-propylphenol, *p*-phenylphenol, *o*-trifluoromethylphenol, *m*-trifluoromethylphenol, *p*-trifluoromethylphenol, *p*-nitrophenol, *p*-nitrobenzoic acid, *p*-hydroxybenzoic acid, and 4,4'-biphenol.

Trends were observed in ECL quenching efficiency of the different phenol derivatives. Most notably, more efficient ECL quenching was observed when substituents were *meta* to the phenol hydroxyl group. For example, *m*-fluorophenol exhibited more efficient ECL quenching as compared to either *o*-fluorophenol or *p*-fluorophenol. Surprisingly, phenol was approximately a factor of 3 more efficient at ECL quenching than any of the phenol derivatives tested.

Example 5

The Effect of Phenol on $Ru(bpy)_3^{2+}$ Photoluminescence.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ was dissolved in Elecsys® buffer solution and diluted to yield a stock solution of 30 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.18 M TPAH (as coreactant) at pH = 6.8. Quantities of 0.2-0.3 M phenol dissolved in ethanol were added to 10 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 0 to 0.3 M. The photoluminescence was measured for each of the samples (with no electrolysis) using a Perkin Elmer LS-50B fluorimeter with the voltage of

the PMT biased at 850 V. Excitation was at 452 nm, the peak maximum of the lowest energy metal-to-ligand charge transfer (MLCT) absorption for the $Ru(bpy)_3^{2+}$ luminophore, with detection between 550 and 650 nm (λ_{em} = 620 nm). The data showed that the photoluminescence increased steadily as the concentration of phenol increased. Note that this trend is opposite to the effect observed for increasing phenol concentration with ECL. Also, the data showed that the effect of phenol on fluorescence was much less dramatic than the effect on ECL.

Example 6

The Effect of Phenol on $Ru(bpy)_3^{2+}$ Photoluminescence: Bulk Electrolysis.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ and TPAH was dissolved in Elecsys® buffer solution and the solution diluted to yield a stock solution of 30 μM $Ru(bpy)_3^{2+}$ and 0.18 M TPAH at pH = 6.8. To a 100 mL aliquot of stock solution was added 6 mL of 1 M phenol, yielding a phenol concentration of 60 mM. A baseline photoluminescence measurement was taken for this initial solution. Controlled potential coulometry (bulk electrolysis) was then performed for 3 hours with continuous stirring using a standard 3-electrode system available from BioAnalytical Systems® Inc. A reticulated vitreous carbon working electrode was biased to an oxidative potential of +1.3 V (versus a Ag/AgCl gel electrode used as reference) to effect electrolysis. A platinum wire counter electrode was separated from the working solution *via* a porous Vycor® frit, and immersed in an appropriate electrolyte solution. During the 3 hour bulk electrolysis, 1 mL samples were taken at ~30 minute intervals for photoluminescence testing (λ_{exc} = 452 nm; λ_{em} = 610 nm), as in Example 5. Approximately 50% of the photoluminescence signal was lost after 2 hr 45 min, indicating that a product of oxidation is directly responsible for photoluminescence quenching. Presumably this product of oxidation is also responsible for the observed ECL quenching.

Example 7

Catechol, Hydroquinone, and 1,4-Benzoquinone Quenching of $Ru(bpy)_3^{+2}$ ECL.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ and TPAH was dissolved in Elecsys® buffer solution and the solution diluted to yield a stock solution of 0.3 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.05 M TPAH (as coreactant) at pH = 6.8. Microliter amounts of 1 M catechol (*i.e.*, 1,2-dihydroxybenzene), hydroquinone (*i.e.*, 1,4-dihydroxybenzene), or 1,4-benzoquinone (all from Aldrich Chemical Company) (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with quenching agent concentrations ranging from 2 to 11 mM. For comparison, microliter amounts of 1 M phenol (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 11 mM. ECL intensity was measured and recorded (in arbitrary units) for each of the samples. The data are illustrated in Figure 4.

This example demonstrates that, at comparable concentrations, catechol, hydroquinone, and 1,4-benzoquinone (the presumed electro-oxidation products of phenol) quench the ECL of micromolar concentrations $Ru(bpy)_3^{2+}$ in the $Ru(bpy)_3^{2+}$/TPAH ECL reaction sequence more efficiently than phenol, with benzoquinone, the most efficient of the three derivatives, being approximately 6 times more efficient than phenol.

Example 8

$Ru(bpy)_3^{+2}$ ECL Quenching by 1,4-Benzoquinone Derivatives.

A number benzoquinone derivatives were tested for their quenching efficiency in a manner analogous to that used in Example 7. Quenching agents were dissolved to the appropriate concentration in ethanol, and appropriate aliquots were transferred to 1 mL aliquots of a $Ru(bpy)_3^{2+}$/TPAH stock solution. Those benzoquinone derivatives which were tested as quenching agents included: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ); 2,5-dibromo-1,4-benzoquinone (BRBQ); 1,2,3,4-tetrafluoro-5,8-dihydroxy-anthraquinone

(TFDAQ); 2-methoxy-3-methyl-1,4-naphthoquinone (MMNQ); and anthraquinone-1,5-disulfonic acid (all >98% purity, from Aldrich Chemical Company).

ECL quenching data for phenol, BQ, and DDQ are shown in Figure 5. DDQ, like many of the benzoquinone derivatives, was approximately a factor of 5 more efficient at ECL quenching than phenol. Benzoquinone was at least a factor of 3 more efficient at ECL quenching than any of the benzoquinone derivatives tested.

Example 9

The Effect of Hydroquinone, Catechol, and Benzoquinone on $Ru(bpy)_3^{2+}$ Photoluminescence.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ was dissolved in Elecsys® buffer solution and diluted to yield a stock solution of 30 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.18 M TPAH (as coreactant) at pH = 6.8. To a 1.2 mL sample of stock solution were added 0.15 mL aliquots of 1 M hydroquinone (Aldrich Chemical Company) dissolved in ethanol. The photoluminescence was measured after each aliquot was added (with no electrolysis and prior to any bulk electrolysis) using the methods described above in Example 5. The data showed an approximately 10% increase in photoluminescence upon the addition of 0.45 mL of the 1 M hydroquinone solution. Note that this trend is opposite to the effect observed for increasing hydroquinone concentration with ECL.

A similar experiment was performed using 1 M catechol (Aldrich Chemical Company) instead of hydroquinone. Surprisingly, the incremental increase of catechol resulted in a decrease in photoluminescence, and approximately 70% of the photoluminescence signal was lost upon addition of 1.2 mL of 1 M catechol.

Another similar experiment was performed using 0.333 M benzoquinone (Aldrich Chemical Company) instead of hydroquinone. Again, the incremental increase of benzoquinone resulted in a decrease in photoluminescence, and approximately 100% of the

photoluminescence signal was lost upon addition of 0.3 mL of 0.333 M benzoquinone. These results clearly demonstrate the efficiency of benzoquinone as a photoluminescence quencher.

Example 10

The Effect of Hydroquinone, Catechol, and Benzoquinone on $Ru(bpy)_3^{2+}$ Photoluminescence: Bulk Electrolysis.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ and TPAH was dissolved in Elecsys® buffer solution and the solution diluted to yield a stock solution of 30 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.05 M TPAH (as coreactant) at pH = 6.8. To a 100 mL aliquot of stock solution was added 6 mL of 1 M hydroquinone (Aldrich Chemical Company), yielding a phenol concentration of 60 mM. Controlled potential coulometry (bulk electrolysis) was performed as described above in Example 6. After 45 minutes, the solution turned a reddish-brown color, indicative of the formation of benzoquinone or some derivative. Also, complete quenching of the photoluminescence was observed within 45 minutes. These data are similar to those observed for phenol, where a substance that enhances $Ru(bpy)_3^{2+}$ luminescence is electrochemically oxidized to form a product that efficiently quenches luminescence.

A similar experiment was performed using 6 mL of 1 M catechol instead of hydroquinone. A complete loss of luminescence was observed within 30 minutes with the concomitant formation of a reddish-brown solution. Although catechol does itself quench photoluminescence at these concentrations (see Example X), the electro-oxidation product of catechol is much more efficient at photoluminescence quenching.

Another similar experiment was performed using 1 mL of 0.333 M benzoquinone instead of hydroquinone. Little or no enhanced photoluminescence quenching was observed upon bulk electro-oxidation. This result is consistent with the conclusion that benzoquinone is responsible for the observed quenching. In fact, a slight increase in photoluminescence intensity was observed, indicating that upon prolonged oxidation, benzoquinone begins to decompose to form non-quenching products.

Example 11

$Ru(bpy)_3^{+2}$/TPAH ECL Quenching by Phenol: Electric Potential Studies.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ and TPAH was dissolved in Elecsys® buffer solution and the solution diluted to yield a stock solution of 0.3 μM $Ru(bpy)_3^{2+}$ (as luminophore) and 0.05 M TPAH (as coreactant) at pH = 6.8. Microliter amounts of 1 M phenol (as ECL quencher) dissolved in ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 6 mM. ECL intensity was measured and recorded (in arbitrary units) for each of the samples using the methods described above, but with potentials of 600, 1000, and 2800 mV in order to assess the degree of ECL quenching and the potentials at which full ECL quenching occurred.

The oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ is known to proceed at +1.3 V versus Ag/AgCl. The oxidation of phenol to products occurs at about + 1.0 V versus Ag/AgCl. As expected, little ECL was observed at potentials of less than + 1.3 V versus Ag/AgCl, since at lower potentials the $Ru(bpy)_3^{2+}$ is not being oxidized. As expected, at higher phenol concentrations and at higher potentials, greater quenching was observed, supporting the conclusion that oxidation of both phenol and $Ru(bpy)_3^{2+}$ are needed for efficient ECL quenching.

Comparative Example 1

$Ru(bpy)_3^{+2}$ ECL Quenching by Methylviologen Carboxylate.

An appropriate amount of $Ru(bpy)_3Cl_2.6H_2O$ was dissolved in Elecsys® buffer solution and diluted to yield a stock solution of 0.3 μM $Ru(bpy)_3^{2+}$ (as luminophore) at pH = 6.8. Microliter amounts of aqueous 10 mM methylviologen carboxylate (MV^{+2}, 1,1-'dimethyl-4,4'-bipyridinium carboxylate dichloride) (as ECL quencher) were added to 1 mL aliquots of stock solution to yield samples with MV^{+2} concentrations ranging from 2 to 10 mM. For comparison, microliter amounts of 1 M phenol (as ECL quencher) dissolved in

ethanol were added to 1 mL aliquots of stock solution to yield samples with phenol concentrations ranging from 2 to 6 mM. ECL was measured for each of the samples, and the ECL intensity (in arbitrary units) recorded. The data are illustrated in Figure 6.

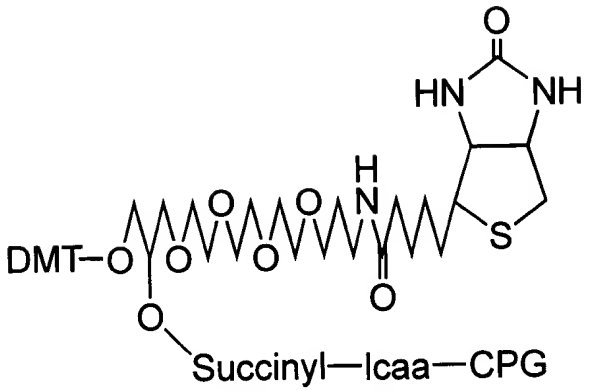
This example demonstrates that, at comparable concentrations, phenol quenches the ECL of micromolar concentrations $Ru(bpy)_3^{2+}$ in the $Ru(bpy)_3^{2+}$/TPAH ECL reaction sequence approximately 10 times more efficiently than methylviologen carboxylate, the "gold standard" of $Ru(bpy)_3^{2+}$ ECL quenching.

Example 12

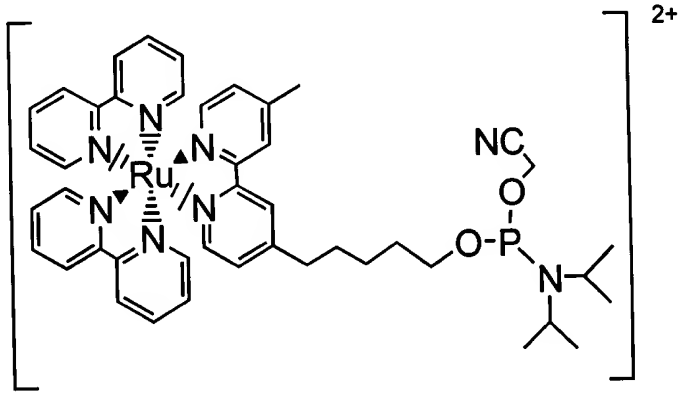
$Ru(bpy)_3^{+2}$/TPAH ECL Quenching by Phenol: Luminophore Immobilized (*via* Magnetic Particles) and Quenching Agent In Solution.

This example illustrates the quenching of an immobilized labeled complex, in this case, an oligonucleotide which was been labeled with the luminophore $Ru(bpy)_3^{+2}$ and subsequently attached to paramagnetic particles, by a quenching agent, in this case phenol, which is present in solution.

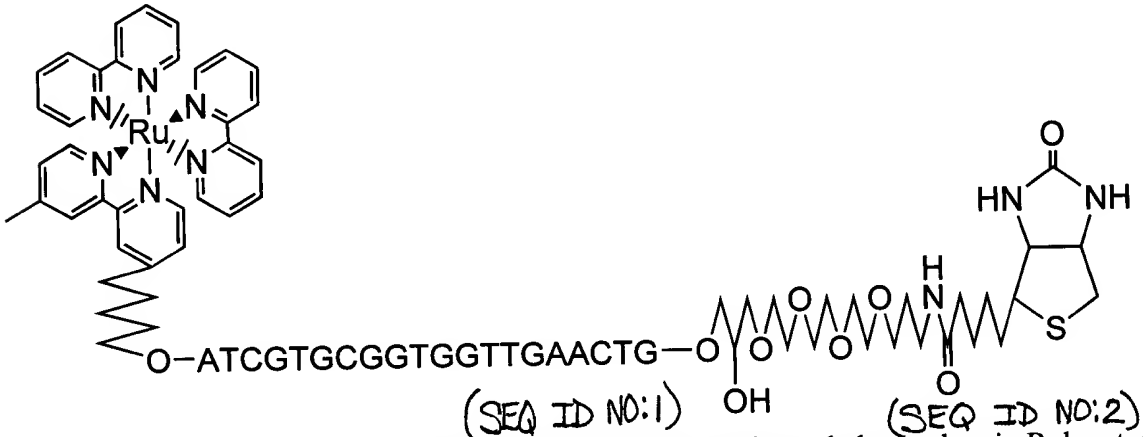
A test oligonucleotide consisting of 20 nucleotide residues was prepared using standard solid phase methods with a Perkin Elmer ABI 394 Synthesizer® using beta-cyanoethyl phosphoramidite chemistry. By using a commercially available (from Glen Research) derivatized controlled pore glass support, illustrated below, which has both a biotin-TEG group and a DMT protected hydroxyl group (*i.e.*, -ODMT), the resulting oligonucleotide possesses, at the 3'-terminus, a tethered biotin group.



The oligonucleotide was synthesized using standard methods. Once the 20-mer oligonucleotide had been synthesized (having a DMT protected 5'-hydroxyl group), a final reaction sequence was performed using the Synthesizer but employing, instead of a nucleotide monomer reagent, a phosphoramidite derivative of a $Ru(bpy)_3^{+2}$, shown below.



In this way, the 20-mer oligonucleotide (shown below) was obtained which possessed, at the 3'-end, a tethered biotin group and, at the 5'-end, a tethered $Ru(bpy)_3^{+2}$ moiety.



This derivatized oligonucleotide may be denoted as shown below, wherein R denotes the $Ru(bpy)_3^{+2}$-containing group and B denotes the biotin-containing group.

5' - R - AT CGT GCG GTG GTT GAA CTG - B - 3'

Superparamagnetic particles (from Dynal Corp., Lake Success, NY) comprising a magnetite (Fe_3O_4) core and a polystyrene outer coating, and having a size of about 2.8 μm, were coated with poly-streptavidin (a protein prepared from the culture supernatant of *Streptomyces avidinii* which has four high affinity binding sites for biotin). A solution of the labeled oligonucleotide was added to a suspension of the streptavidin-coated magnetic beads to yield labeled magnetic beads having bound labeled oligonucleotide.

Since phenol was to be added to some samples, a buffer solution was prepared based on Elecsys® buffer solution and formulated to have 27.19 g/L potassium phosphate monobasic (KH_2PO_4); 0.2 g/L Triton X-100 (*t*-octylphenoxy-polyethoxyethanol); and 0.05 M TPAH. The pH of the buffer solution was adjusted to 7.0 with 4M aqueous NaOH. For those cases where phenol was to be added to the buffer solution, 1.2 μL of 1 M phenol dissolved in ethanol was added to give 1 mM phenol and the pH again adjusted to 7.0 with 4 M aqueous NaOH.

A 3 μL aliquot of a suspension of labeled magnetic beads (546 pmol $Ru(bpy)_3^{+2}$ label) was added to 1 mL of buffer solution to yield a working bead solution. This working bead solution was placed in the ECL cell and the labeled magnetic particles immobilized onto the surface of the working electrode. Buffer solution containing coreactant (and, in some cases, phenol) was swept into the cell, and an appropriate potential is applied to generate signal. ECL was measured and recorded (in arbitrary units) for five control cases (using buffer without phenol) and ten quenching cases (using buffer with added phenol). In the control cases, ECL signals of about 75,000 arbitrary units were observed from the labeled beads in the absence of phenol. Virtually no ECL signal was observed for the labeled beads in the presence of phenol.

This example clearly demonstrates that in a magnetic bead format where the ECL luminophore is immobilized (in this case, attached to an oligonucleotide, and the oligonucleotide attached to a magnetic bead), quenching of the ECL luminophore still occurs.

Example 13

$Ru(bpy)_3^{+2}$/TPAH ECL Quenching by a Benzoquinone: Both Luminophore and Quenching Agent Immobilized (*via* Magnetic Particles).

This example illustrates the quenching of an immobilized labeled complex, in this case, an oligonucleotide which was been labeled with the luminophore $Ru(bpy)_3^{+2}$ and attached to paramagnetic particles, by a quenching agent, in this case a benzoquinone, which has also been attached to the oligonucleotide.

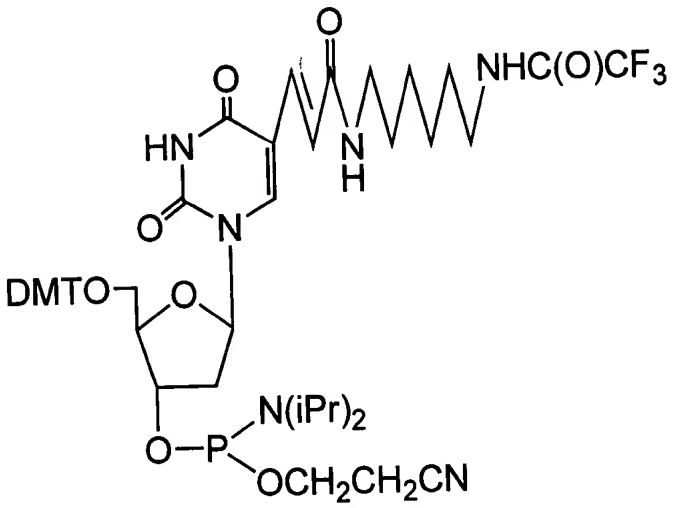
Three oligonucleotides consisting of 21 nucleotide residues were prepared using standard solid phase methods with a Perkin Elmer ABI 394 Synthesizer® using beta-cyanoethyl phosphoramidite chemistry, in a manner analogous to that described in Example 12. The three resulting derivatized oligonucleotides are illustrated below (SEQ ID NOS:3-5) where R denotes the $Ru(bpy)_3^{+2}$-containing group and B denotes the biotin-containing group.

5' - R - CAG ***T***TC CAA CCA ACC GCA CGT - B - 3' (13-1-R)

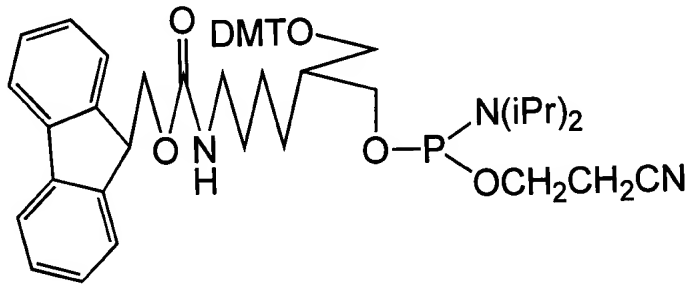
5' - R - CAG TTC CAA CCA ACC GCA CG***T*** - B - 3' (13-2-R)

5' - R - CAG TTC CAA CCA ACC GCA CGT ***LLLLL*** - B - 3' (13-3-R)

In the above formulae, ***T*** denotes "amine modified C_6-dT," a commercially available (from Glen Research) modified thymine nucleotide residue, illustrated below, which was introduced during oligonucleotide synthesis.



Also in the above formulae, ***L*** denotes "Label On," a commercially available (from Glen Research) reagent, illustrated below, which permits that attachment of common labels and which was introduced during oligonucleotide synthesis.



The above three derivatized oligonucleotides (each having a $Ru(bpy)_3^{+2}$ group) were employed as controls. Three test oligonucleotides, shown below (SEQ ID NOS:6-8), were prepared by attaching a benzoquinone moiety was at the position marked ***T*** and at each position marked ***L***.

(13-1-RQ)

5' - R - CAG ***T(Q)***TC CAA CCA ACC GCA CGT - B - 3'

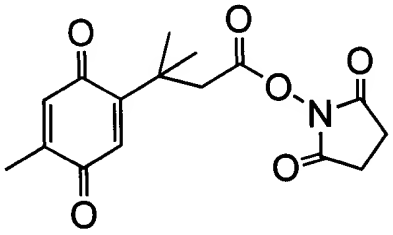
(13-2-RQ)

5' - R - CAG TTC CAA CCA ACC GCA CG***T(Q)*** - B - 3'

(13-3-RQ_5)

5' - R - CAG TTC CAA CCA ACC GCA CGT ***L(Q)L(Q)L(Q)L(Q)L(Q)*** - B - 3'

For ***T***, the protected amine group (*i.e.*, -NHC(=O)CF_3) was first deprotected, and the free amine group subsequently reacted with the N-succinimidyl ester of a benzoquinone derivative, shown below. For ***L***, the protected amine group (*i.e.*, -NHFMOC) was first deprotected, and the free amine was subsequently reacted with the same N-succinimidyl ester of the benzoquinone derivative.



The three test oligonucleotides (and their standards) were quantified by UV-VIS absorption spectroscopy, using the absorbance of the $Ru(bpy)_3^{2+}$ moiety at 456 nm ($\varepsilon = 13000\ M^{-1}\ cm^{-1}$) so that equivalent amounts of each test oligonucleotide could be used for ECL analysis. In a manner analogous to that in Example 12, suitable amounts of each test oligonucleotide were added to suspensions of the streptavidin-coated magnetic beads to yield labeled magnetic beads having bound labeled oligonucleotide such that 1/3 saturation of streptavidin sites was achieved (576 pmol Biotin/DNA labeled probes).

Each labeled magnetic bead suspension was examined. A 3 µL aliquot of the labeled magnetic bead suspension was placed in 1 mL of Elecsys® buffer solution to yield a working bead solution. This working bead solution was placed in the ECL cell of the Origen analyzer and the labeled magnetic particles immobilized onto the surface of the working electrode. Elecsys® buffer solution containing coreactant was swept into the cell, and an appropriate potential was applied to generate signal. ECL was measured and recorded (in arbitrary units) for five replicates for each of the four test oligonucleotides.

For test oligonucleotide 13-1-RQ, in which the $Ru(bpy)_3^{2+}$ luminophore is separated from the quenching benzoquinone group by 4 nucleotide residues, the observed ECL intensity was approximately 53% less than that observed for the control oligonucleotide 13-1-R which has no quenching moiety.

For test oligonucleotide 13-2-RQ, in which the $Ru(bpy)_3^{2+}$ luminophore is separated from the quenching benzoquinone group by 21 nucleotide residues, the observed ECL intensity was approximately 49% less than that observed for the control oligonucleotide 13-2-R which has no quenching moiety.

For test oligonucleotide 13-3-RQ_5, in which the $Ru(bpy)_3^{2+}$ luminophore is separated from the (five) quenching benzoquinone groups by 21 nucleotide residues, the observed ECL intensity was approximately 20% less than that observed for the control oligonucleotide 13-3-R which has no quenching moiety.

This example clearly demonstrates that in a magnetic bead format where both the ECL luminophore and the quenching moiety are immobilized (in this case, both are attached to an oligonucleotide, and the oligonucleotide attached to a magnetic bead), quenching of the ECL luminophore still occurs.

Example 14

$Ru(bpy)_3^{+2}$/TPAH ECL Quenching by a Benzoquinone: Restriction Enzyme Methods

This example illustrates the use of restriction enzymes coupled with bead capture and subsequent ECL detection. In this case, oligonucleotide hybridization probes are labeled with $Ru(bpy)_3^{2+}$ and biotin at the 3'-terminus and a quenching moiety at the 5'-terminus. Two pairs of oligonucleotides (SEQ ID NOS 9-12) having tethered biotin groups at the 3'-terminus are synthesized using standard solid phase methods as described in Example 12.

5' - *N*AC GCC ACT GGA TCC ACA GTT AG*Tc* - B - 3' (14-A-1)

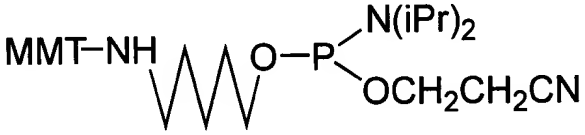
5' - AAC GCC ACT GGA TCC ACA FTT AG*Tc* - B - 3' (14-A-2)

5' - *T* TTG CGG TGA CCT AGG TGT CAA TCA *Tc* - B - 3' (14-B-1)

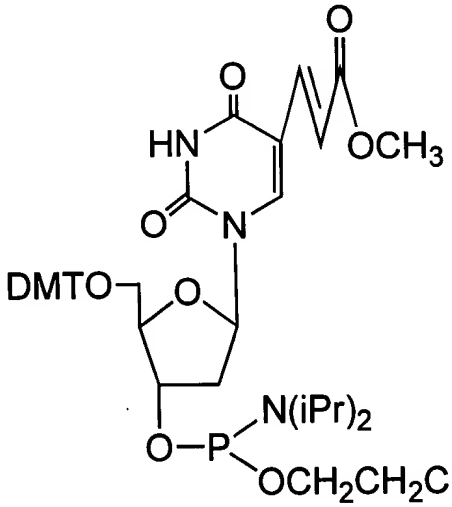
5' - TTG CGG TGA CCT AGG TGT CCA TCA *Tc* - B -3' (14-B-2)

Each pair pertains to a separate example and each member of a given pair comprises the same specific probe sequence which is complementary to a sequence in a DNA target to be detected. The second member of each pair, which will not be derivatized to have a quenching moiety, is used for comparison purposes to verify quenching; the second member provides an indication of the ECL emission in the absence of a quenching moiety. The underlined residues, CCT AGG, identify part of the BamHI enzyme restriction site, as discussed below.

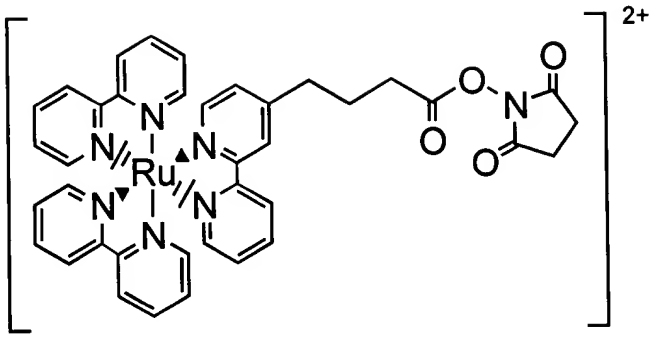
In the above formulae, ***T*** is as defined above in Example 13 and ***N*** denotes "5'-amino modifier," a commercially available (from Glen Research) reagent, illustrated below (where MMT is 4-monomethoxytrityl) which was introduced during oligonucleotide synthesis.



Also in the above formulae, ***Tc*** denotes "carboxy modified dT," a commercially available (from Glen Research) modified thymine nucleotide residue, illustrated below, which was introduced during oligonucleotide synthesis.



Following cleavage of the oligonucleotides from the solid phase support, a $Ru(bpy)_3^{2+}$ group is covalently attached at the 3'-terminus by reacting the following N-hydroxysuccinimidyl ester derivative with the carboxy group of ***Tc***.



A quenching moiety is then covalently attached at the 5'-terminus of first member of each pair *via* the amino groups of ***N*** and ***T***, using the activated benzoquinone derivative illustrated in Example 13. In this way, the following two pairs of derivatized oligonucleotide hybridization probes (SEQ ID NOS: 13-16) are obtained. The ECL emission of the first member of each pair is quenched by the presence of the quenching moiety, as illustrated by comparison with the ECL emission of the corresponding second member, which has no quenching moiety.

5' - ***(Q)N***AC GCC ACT GGA TCC ACA GTT AG***Tc***(R) - B - 3' (14-A-1-BRQ)

5' - AAC GCC ACT GGA TCC ACA FTT AG***Tc***(R) - B - 3' (14-A-2-BR)

5' - **(Q)*T*** TTG CGG TGA CCT AGG TGT CAA TCA ***Tc***(R) - B - 3' (14-B-1-BRQ)

5' - TTG CGG TGA CCT AGG TGT CCA TCA ***Tc***(R) - B -3' (14-B-2-BR)

The first member of a pair of derivatized oligonucleotide hybridization probes (*e.g.*, 14-A-1-BRQ or 14-B-1-BRQ) is then added to a sample containing single stranded DNA. The derivatized oligonucleotide probe will hybridize only with the complementary target sequence. The restriction enzyme *BamH1* is added. This enzyme recognizes only a specific double stranded DNA sequence, as shown below.

←5' - GGA TCC - 3' →

←3' - CCT AGG - 5' →

The restriction enzyme cleaves this sequence between the GG residues to yield two fragments, each with a 5'-overhang, as shown below.

←5' - G 5' - GA TCC - 3' →

←3' - CCT AG - 5' G - 5' →

In this way, each target DNA sequence leads to a cleavage event, and the formation of a cleavage fragment which possesses an ECL label no longer in quenching contact with an ECL quenching moiety. These cleavage fragments also possess a biotin group (as well as an unquenched ECL label), which permit their capture (and optional separation) with the aid of streptavidin coated magnetic beads, as in Example 12. The ECL emission is then measured and correlated with the amount of target DNA in the original sample. Of course, bead capture may be performed prior to or after enzymatic cleavage.

Again, for comparison purposes, ECL emission is measured following hybridization and prior to enzymatic cleavage for the first and second members of a given pair. When the probe has hybridized with the target DNA, the ECL emission of the first member of each pair remains quenched by the presence of the quenching moiety, as illustrated by comparison with the ECL emission of the corresponding second member, which has no quenching moiety.

F. References

The disclosures of the publications, patents, and published patent specifications referenced below are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

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